CRANFIELD UNIVERSITY

Emma Butler

# The fate and effects of triclosan in soils amended with biosolids

School of applied Science
Department of environmental science and technology

PhD
Academic Year: 2008 - 2012

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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD

# ABSTRACT

Many hydrophobic pollutants can be emitted to agricultural soils if sewage sludge is used as a fertiliser. The fate and effects of pollutants in such receiving environments are relatively poorly understood compared with our knowledge of chemical behaviour and impact in surface waters. One chemical of particular concern is triclosan because it has antimicrobial properties which could affect important soil functions. Triclosan is hydrophobic, which means it will sorb appreciably to organic solids and is not readily biodegradable. It is also used extensively in personal care products. These factors have prompted considerable attention in the literature with respect to its environmental profile. In recent years, this attention has shifted away from the water environment to terrestrial systems. This thesis bridges some of the knowledge gaps considering the fate (specifically mineralisation, primary degradation and the formation of bound residues) and the effects (to the soil function and phenotype) of triclosan in soils amended with biosolids.

The fate of triclosan in biosolid amended soils was studied in both laboratory and field scale studies. The laboratory based incubation study used radio-labelled triclosan to trace the fate of triclosan in soils and biosolid amended soils over time, whereas the field study looked at triclosan when sludge is applied to agricultural land. Firstly it was observed that triclosan was very slow to mineralise with less than 7% mineralisation observed after 6 months. It was also seen that the addition of triclosan to the soil via biosolid application resulted in slower mineralisation than when triclosan was directly applied to the soil. Two of the key findings however were:

1. The rapid formation of the metabolite methyl-triclosan in both laboratory and field scale studies. At the end of both studies the majority of the applied compound (> 50 %) was in the methylated form. Conversely, methyl-triclosan did not degrade during the time of the study resulting in almost a complete mass balance of applied test compound.
2. Bound residues were also rapidly formed and could only be extracted using harsh solvents (in the field study) as the gentler approach tried in the laboratory study struggled to extract more than 36% of the applied triclosan.

In addition to looking at triclosan fate, the effects were also studied. The endpoints of respiration inhibition and biomass changes were used to determine the effect on microbial function, whereas changes in fatty acid phospholipid composition were analysed to determine the impact of triclosan of the phenotypic community structure. With regards to the functional changes it was observed that triclosan did inhibit both respiration and biomass and that the inhibition was proportional to the nominal dose applied. However, both respiration and biomass recovered to levels equal to or greater than that measured in the control soils suggesting that the soils were resilient to triclosan dosing. By also studying phenotypic changes, it enabled a better understanding into whether the observed recovery of microbial respiration and biomass and the different response to re-dosing was due to microbial acclimation or due to changes in the microbial community structure. A temporal shift in the soil community structure was observed, however the shift is also observed in the control soils suggesting that the initial disturbance of the soil prior to the addition of triclosan could have been influential in this shift. There was still a definite dose related triclosan effect.

# ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Dr Michael Whelan and Dr Ruben Sakrabani for their continuous support, encouragement, advice and assistance throughout this PhD.

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# TABLE OF CONTENTS

# LIST OF FIGURES

LIST OF TABLES

# Chapter 1

## Introduction and thesis layout

## 1.1 Scope

Many everyday consumer goods such as home and personal-care products e.g. soap, shampoo, laundry detergents and toothpaste products, contain chemical ingredients which, as a consequence of their normal usage, can be introduced into the environment. Most of these products are discharged down the drain during and after use. In most developed countries waste water is treated in wastewater-treatment plants where chemicals can be removed by degradation (biodegradation, hydrolysis and photolysis), volatilisation or by sorption to sludge solids (Figure 1-1). Chemical fate will depend on a compounds physical and chemical properties, its biodegradability and on the waste-water treatment processes employed. In most cases, a part of the input load is not removed and this will be discharged into the environment. There are three main pathways for environmental emission (Figure 1-1): volatilisation into the atmosphere, discharge to the aquatic environment via treated effluent and emission to the terrestrial environment through the addition of sludge-sorbed chemical to land. Until relatively recently the research focus has been principally directed at the fate and effects of these so called “down the drain” chemicals in waste-water and the aquatic environment. The fate and effects of synthetic organic pollutants in soils receiving sewage sludge has been relatively poorly studied, although there is now considerable interest in this pathway. There is particular interest in compounds with antimicrobial properties because of their potential to disrupt important microbially mediated soil functions such as nitrification. This thesis explores aspects of the fate and effects of a commonly used antimicrobial compound triclosan. This chapter highlights the context and the rationale for the work which follows, describes the specific objectives and the thesis

structure. Some of the literature on the fate and effects of triclosan in the environment has been reviewed in this chapter, although not exhaustively as targeted reviews are included in the individual chapters which follow.

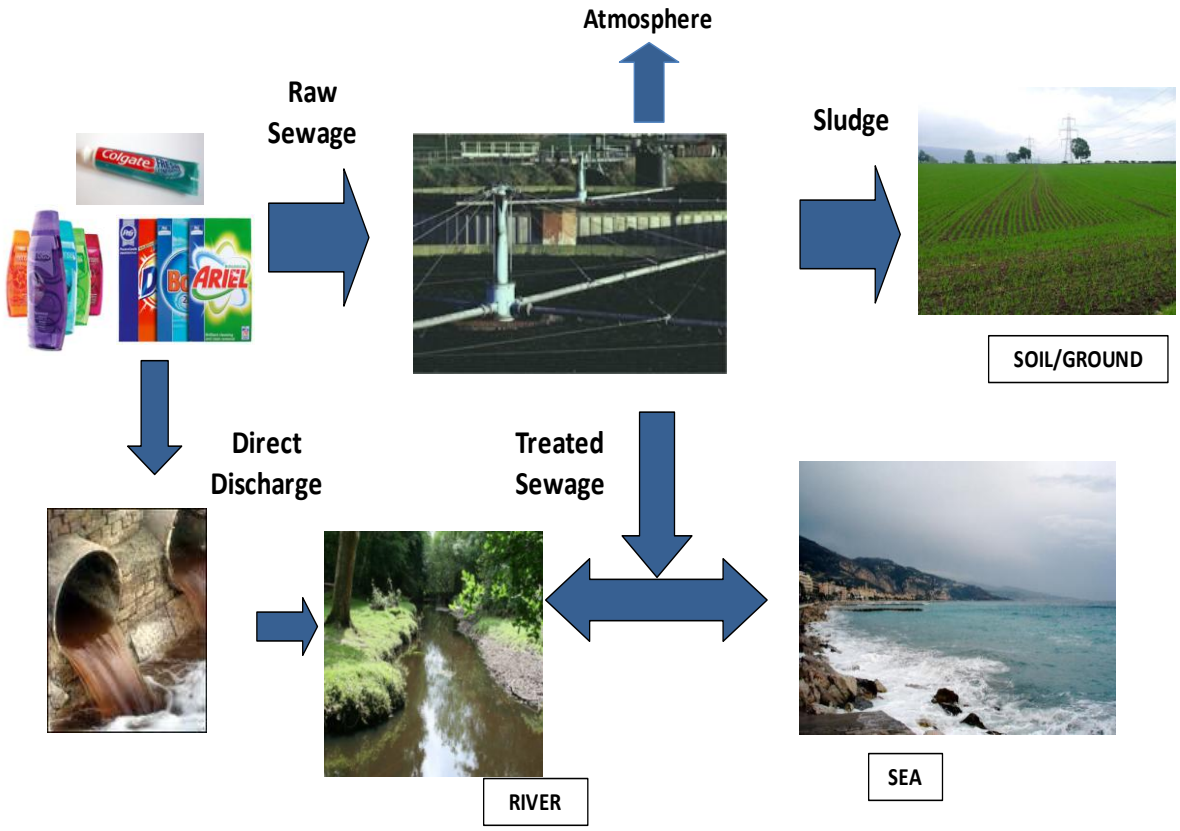



**Figure 1-1Schematic illustration of the environmental emission pathways for "down the drain" chemicals after use.**

## 1.2 Background

Ingredients in home and personal-care products include complex organic molecules with different functionalities, and chemical and biological properties. A product such as shampoo or washing powder can be seen to contain between 10 and 20 active ingredients (Kümmerer, 2011), all with the potential to be rinsed down the drain, into the wastewater treatment plant and ultimately, into the environment. There are several potential fate pathways that the compound can take including degradation, transformation, volatilisation and sorption to solids. Some molecules can remain unchanged and are emitted into the aquatic environment where, once again, they can be subjected to different fate processes depending on the nature of the compound and the receiving environment (Figure 1-2).

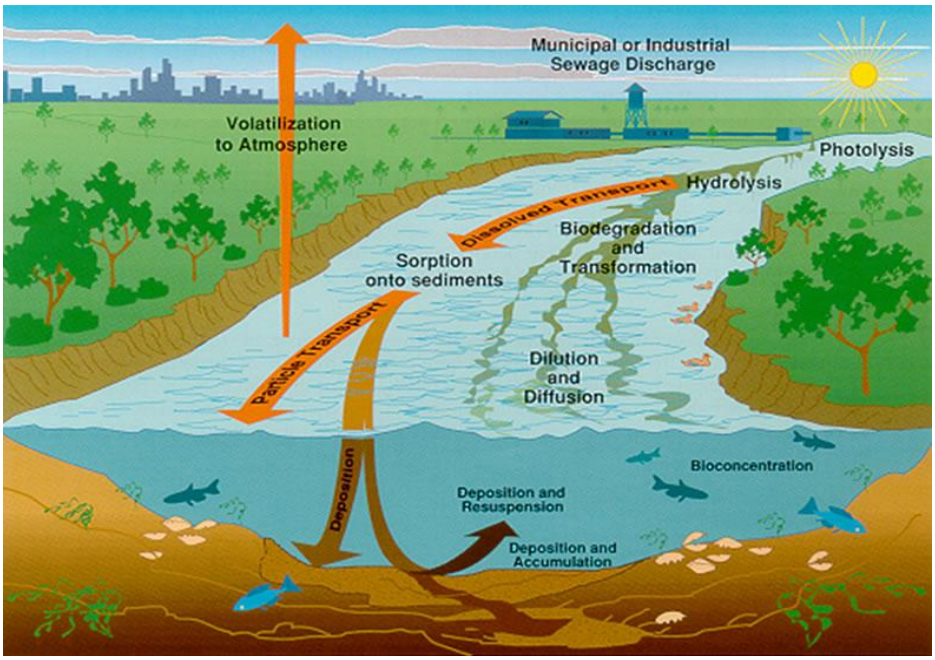



**Figure 1-2 The fate of pollutants in the aquatic environment (USGS, 2012).**

**The wastewater treatment process**

Most sewage treatment plants employ three stages of treatment (Figure 1-3):

1. A physical barrier to remove large solid matter and grit from the water
2. Settlement tanks to remove suspended solids from the water
3. Use of microorganisms to digest and breakdown organic substances

The microorganisms are either present as a fixed biofilm on solid surfaces (Lewandowski and Boltz., 2011) and the waste water is trickled through the solids (trickling filter system: TF) or are suspended in the waste-water (mainly sorbed to suspended particles) in activated sludge systems (which involve the addition of aerated agitated liquor: Warner *et al*., 1986). In some instances a tertiary treatment stage is also used (e.g. chlorination and UV treatment or a combination of both: Oppenheimer *et al*, 2007), which can involve disinfection of treated effluent and or the removal of nitrate and phosphorus before discharge. Recent trials have also used ozone treatments with some success (Nakada *et al*, 2007).

As well as emission of treated effluent, solids are also removed from the process as sewage sludge. Sewage sludge is disposed of via a number of routes (Figure 1-5) including application to land in agriculture (Sabourin *et al*., 2009) and as an energy source via incineration or digestion (Roy *et al.,* 2011) or simply to landfil.

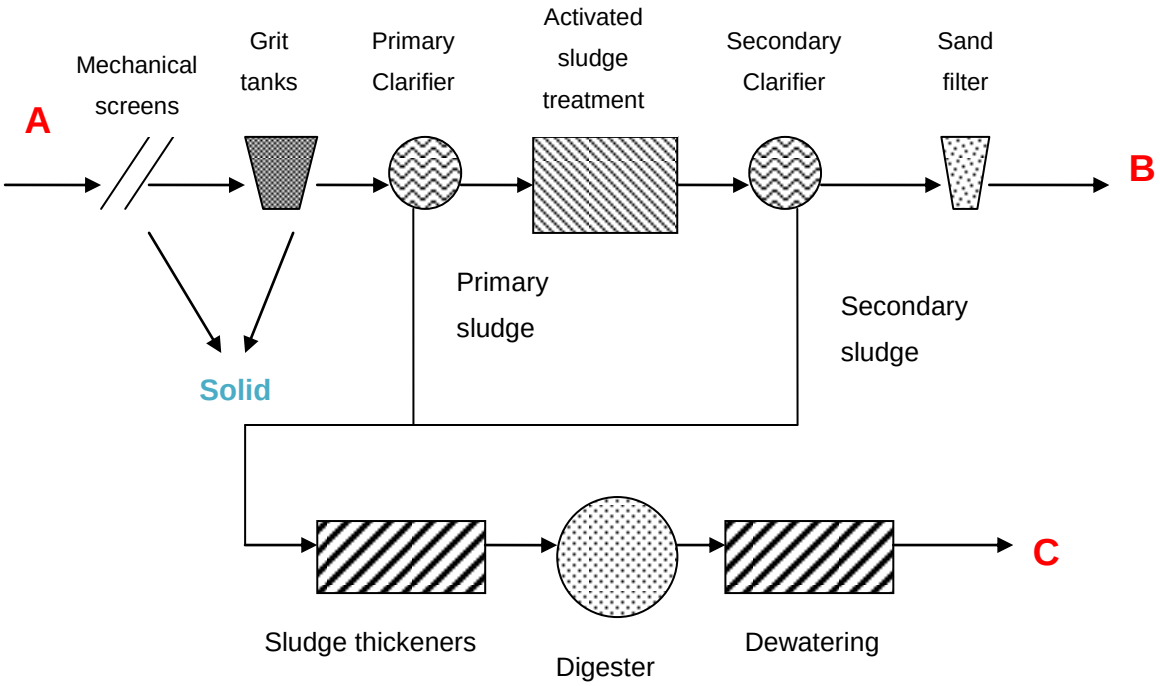



**Figure 1-3: A basic schematic of a wastewater treatment plant where A is the influent, B is the effluent and C is the dewatered sludge (Adapted from Heidler and Halden, 2007).**

### 1.2.1 Sewage sludge treatment process

Sewage sludge is produced from the treatment of wastewater and consists of two basic forms; raw primary sludge and secondary activated sludge. Primary sludge is basically faecal matter whereas the secondary activated sludge has been through the treatment process and may have a different particle size distribution and associated microbial community that will degrade organic contaminants from wastewater using them primarily as an energy source but also via secondary metabolism. The sewage sludge is finally treated to form “biosolids” using a number of complex treatments such as digestion, lime stabilisation, thickening, dewatering and drying (Fig 1-4) to create various product types.

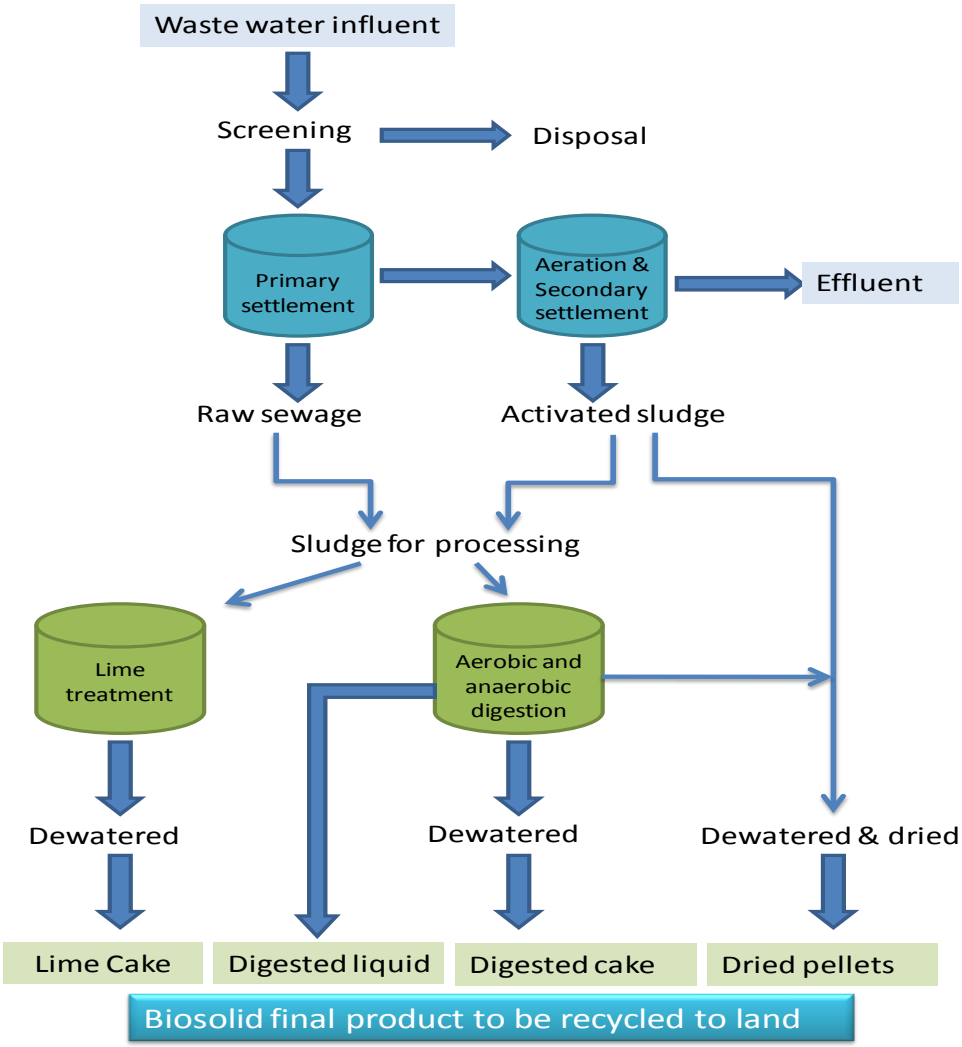



**Figure 1-4 A flow diagram detailing the formation process of the different forms of biosolids. (Adapted from Water UK, 2010):**

### 1.2.1.1 The effects of sludge treatment on contaminant behaviour

The addition of $CaCO_3$ to sewage sludge (liming) is used to help kill pathogens and to maintain optimum pH for soil application as well as to reduce the solubility of trace and heavy metals reducing their bioavailability (Jamali *et al*., 2008). This can also have a negative effect on the utility of the sludge for agriculture as liming also reduces the bioavailability of nutrients such as manganese and phosphate in the soil

(Evans *et al*., 1995). As well as affecting soil fertility, pH also plays an important role in the fate of many organic ionisable pollutants because it controls the fraction of compound in neutral and ionised form- which in turn, controls mobility and bioaccumulation. For example the flame retardant tetrabromobisphenol A (TBBPA) was observed to have decreased sorption to soil with an increase in soil pH making it more mobile and more bioavailable in limed soils (Sun *et al*., 2008).

Sewage sludge post-treatment to has been shown to influence soil microbiological activity, especially basal respiration. These changes tend to occur in the first few weeks following the surface application of the sludge. For example thermally dried sludge mineralises quickly, releasing ammonium and nitrates (Fernandez *et al.*, 2006), causing a rapid increase in microbial biomass and activity. (Jimenez *et al*., 2007). In contrast composted sludge can be used as an organic amendment that is more stable and longer lasting (Tarrason *et al*., 2010) i.e. it mineralises less readily.

Liquid biosolids that have not undergone a dewatering step can rapidly mobilise soil contaminants resulting in the leaching of nutrients and pollutants into ground water and tile drains (Lapen *et al*., 2008). In addition liquid sludge applied to unsaturated soil will fill available pore space, ensuring greater exposure of soil microorganisms to associated contaminants (Sabourin *et al.,* 2009). Thermally dried biosolids sometimes have high density aggregates with low pore volume which can restrict the diffusion of oxygen and thereby, enhance pollutant persistence (Edwards *et al.,* 2009).

### 1.2.2 Biosolids for agricultural use

Over 9 million tonnes of biosolids are produced annually in the EU; more than 1.35 million tonnes of this amount is generated in the UK, which is the 2nd highest producer after Germany (Magoarou, 2000). Everyday, each person in the UK produces approximately 55g of biosolid (dry mass) equating to 20 kg per annum (Water UK, 2006). Over 62% of sludge produced in the UK is spread on agricultural land, 19% is incinerated, 11% used in land reclamation and the remainder is used in composting, industry or disposed as landfill (Figure 1-5: Water UK, 2006). The disposal of biosolids to sea was banned by the European Union (EU) Urban Waste Water Treatment Directive (91/271/EEC), which came into force in 1998.

The usage is higher in the UK than the EU average (36.4%) and use in the USA (41.0%) (Renner, 2000). There are several advantages to biosolid use on land. Firstly, it is the low cost option of sludge disposal compared to incineration (Laturnus *et al.*, 2007). However, sludge has high organic matter and nutrient content and, as a result can also improve soil quality. Increased organic matter can improve soil structure, soil moisture retention, decrease bulk density and increase soil porosity (Ojeda *et al.*, 2003). It is also widely accepted that biosolid application can produce higher crop yields due primarily to the high nutrient levels in the sludge (Petersen *et al.,* 2003, Wang, 2006). Currently in the UK, biosolids only represent 5% of the current organic fertilisers used; approximately 90% still comes from animal manure and abattoir waste (Water UK, 2006).

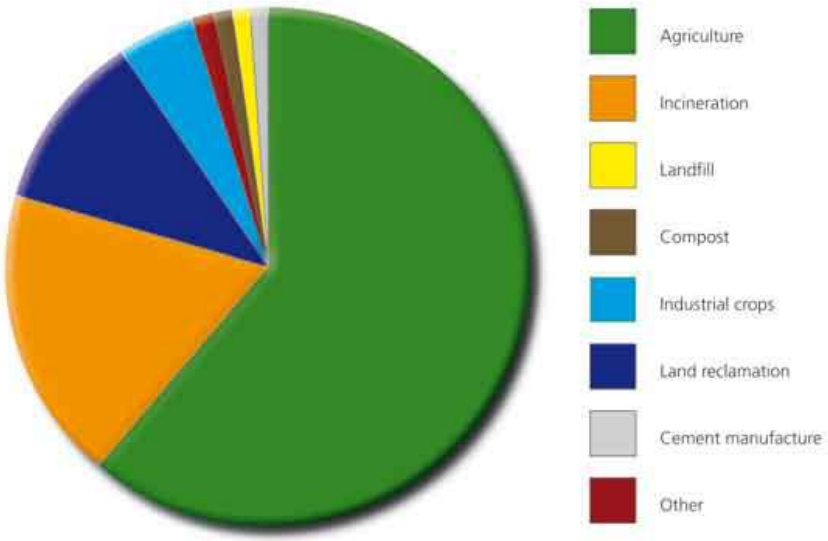



**Figure 1-5 Uses of sewage sludge in the UK (Water UK, 2006)**

### 1.2.2.1 Legislation - The Safe Sludge Matrix

There is a strict legal framework in place to regulate the recycling of biosolids. The current controls originate from the 1986 EU sludge Directive (86/27/EEC) and the UK sludge (use in agriculture) regulations 1989 (SI 1989, no 1263). Other regulations also govern the use of biosolids, such as the Code of Practice for agricultural use of sewage sludge (DoE, 1990) and the Code of good agricultural practice for the Protection of Water (MAFF, 1998). Legislation in the EU, stipulates that treated biosolids must ideally be free from conventional pollutants such as heavy metals. A voluntary agreement, known as the "Safe Sludge Matrix" has been in place throughout the UK since 31st December 1998. The agreement, made between Water UK, sewage operators and the British Retail Consortium (BRC), invoked a revision to the Sludge (use in Agriculture) regulations 1989 and has

influenced the ongoing revision of the EU Sludge Directive. The following organisations were involved in the development of the safe sludge matrix; DEFRA (Department of Environment, Farming and Rural Affairs) and the Food Standards Agency (FSA), National Farmers Union (NFU), Country Land and Business Association (CLA), food manufacturers and food processors. The matrix sets out controls for the quality of the sludge applied and outlines the correct procedures which should be followed.

#### 1.2.2.2 Organic pollutants present in biosolids

Biosolids contain nutrients such as nitrogen, phosphorus, potassium and organic matter, which can benefit soil properties and promote plant growth (Chitdeshwari *et al.,* 2001). Biosolids can, however, also contain undesirable contaminants such as pathogens, heavy metals and organic pollutants, at varying concentrations depending on the pollutant type, wastewater provenance and the wastewater treatment facilities at which the solids are produced (Zitomer and Spreece, 1993). Sorption is the major contributory factor for the presence of contaminants in sludge. For example, even extremely volatile compounds such as benzene can be found in sludge due to a moderate affinity for the high levels of organic matter present in the sludge matrix (Wild *et al.,* 1992). Once the pollutants have been sequestered into sludge they will degrade much more slowly than in the liquid effluent as the microstructures in the biosolid can provide some physical protection for the compounds (from microbial attack for example). In addition, there maybe some interaction with complex organic macromolecules, such as humic and fulvic

substances, leading to the formation of reversible or irreversible residues (e.g. bound residues). This means that degradation and diffusion in the sludge can be very slow (Hatzinger and Alexander, 1997).

## 1.3 Triclosan

Triclosan (5-Chloro-2-[2, 4-dichloro-phenoxy]-phenol) is a broad spectrum antimicrobial agent. It possesses mostly antibacterial properties, affective against both Gram positive and Gram negative bacteria (Schweizer, 2001), but also some antifungal (Hoq *et al.,* 2008) and antiviral properties (Tierno, 1999). Triclosan is used in several home and personal-care products such as toothpastes, detergents, soaps, cosmetics, deodorants and mouthwashes (Dayan, 2007) under several trade names including IRGASAN DP 300 and IRGACARE MP (Table 1-1) at concentrations ranging between 0.1 – 1% (w/w) (Ciba speciality chemicals, 1998). Triclosan is also commonly incorporated into polymers and fibres to make clothes, shoes, food chopping boards and sportswear (Bhargava and Leonard, 1996; Jones *et al*., 2000) under the brand name of Microban® and is also added to acrylic fibres under the trade name Biofresh®.

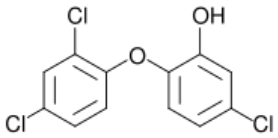
In the EU, about 85% of the total volume of triclosan is used in personal care products, compared to 5% for textiles and 10% for plastics and food contact materials (SCCS, 2010). Due to its antimicrobial properties, triclosan is used all over the world with an estimated annual usage in excess of 350 tonnes in the European Union alone (Singer *et al.*, 2002), although it is presumed that this amount has

significantly increased with the increase in the size of Europe over the past decade. It was reported that in Sweden a quarter of all toothpastes contained triclosan which equated to over 2 tonnes of triclosan per annum (Adolfsson-Erici *et al*., 2002). Capdeveille *et al*. (2008) assumed a per capita usage of 1g/cap/year in their aquatic risk assessment, which is equivalent to 60 tonnes per year in the UK.

### 1.3.1 Triclosan physico-chemical properties

Triclosan is a white powdered solid with a slight aromatic/ phenolic odour. It has both ether and phenol functional groups. Phenols often naturally present with antibacterial properties (Park *et al.,* 2001). Triclosan is only slightly soluble in water, but soluble in many solvents such as methanol, ethyl-acetate and diethyl ether. In addition, due to its weak acidity, it is also soluble in strong basic solutions such as sodium hydroxide (Ciba speciality chemicals, 2001).

**Table 1-1: Chemical properties of Triclosan**

| | | |
|---|---|---|
| Triclosan structural formula |  | Sigma-Aldrich |
| Molecular formula | $C_{12}H_7Cl_3O_2$ | Sigma-Aldrich |
| Trade names | Irgasan® DP300, Irgasan® PG60, Irgacare® MP, Irgacare® CF100, Irgacide® LP10, ; Cloxifenolum, Irgagard® B 1000, Lexol 300, Ster-Zac | SCCS, 2010 |
| Molecular mass (g $mol^{-1}$) | 289.5 | Sigma-Aldrich |
| Melting point (˚C) | 56 | Sigma-Aldrich |
| Water solubility (mg $L^{-1}$) | 10 (20˚C) | McAvoy *et al*., 2002 |
| Dissociation constant (pKa) | 8.14 (20˚C) | Reiss, 2009 |
| Vapour pressure (Pa) | 1.84 $X10^{-4}$ | Beyer *et al*., 2002 |
| Log $K_{OW}$ | 4.8 | Beyer *et al.,* 2002 |
| Log $K_{OC}$ (L $kg^{-1}$) | 4.3 | Seth *et al*., 1999 |
| H (Pa $m^3$/mol) | 0.0023 | Beyer *et al*., 2002 |

**Log Kow is the partitioning coefficient of the chemical between octanol and water, Koc is the organic carbon partition coefficient and H is the Henry's law constant.**

## 1.3.2 Triclosan toxicity to humans

Triclosan enters the human body orally through toothpaste, mouthwashes and dental treatments although it shows no toxicity to humans and is not an irritant to the skin

and eyes when used normally. This makes it appealing as an antimicrobial agent for these uses. Triclosan is neither carcinogenic, mutagenic nor teratogenic and is easily metabolised in the body into its glucuronide or sulphate conjugates (Figure 1-6), which, according to the principal manufacturer, BASF (formally Ciba), are non toxic and do not bioaccumulate in tissues or organs due to elimination from the body (Ciba speciality chemicals, 1998). Other metabolites have also been detected in rats such as; Monohydroxylated triclosan, 2,4-Dichlorophenol and 4-Chlorocatechol (Aguillon et al., 2010). On the other hand, triclosan has been detected in the human body in some studies. For example, high levels were found in 3 out of 5 randomly selected samples of human breast milk, chosen to represent the general population, by Adolfsson-Erici *et al.* (2002) with levels as high as 300 µg $kg^{-1}$ (lipid weight). Triclosan has also been identified in 100% of 70 human plasma samples tested and half of the breast milk samples, all of which were above levels of quantification (18 µg $kg^{-1}$ in milk samples and 9 µg $kg^{-1}$ in plasma) (Allmyr *et al.,* 2006) and 75% of 2500 urine samples (Calafat *et al.,* 2008). Triclosan was also detected at concentrations as high as 2100 µg $kg^{-1}$ in all but two of the Sixty two breast milk samples supplied to a milk bank in the USA were tested and samples (Dayan, 2007).

Although triclosan has low acute oral and dermal toxicity, it has been observed that there is some evidence respiratory complications through repeated inhalation (NICNAS, 2008). A study looking at the effect of using a triclosan based toothpaste (0.03%) on thyroid activity over a four year period, showed that there were no

observed effects despite triclosans structural similarity to thyroid hormones (Cullinan *et al*., 2012).

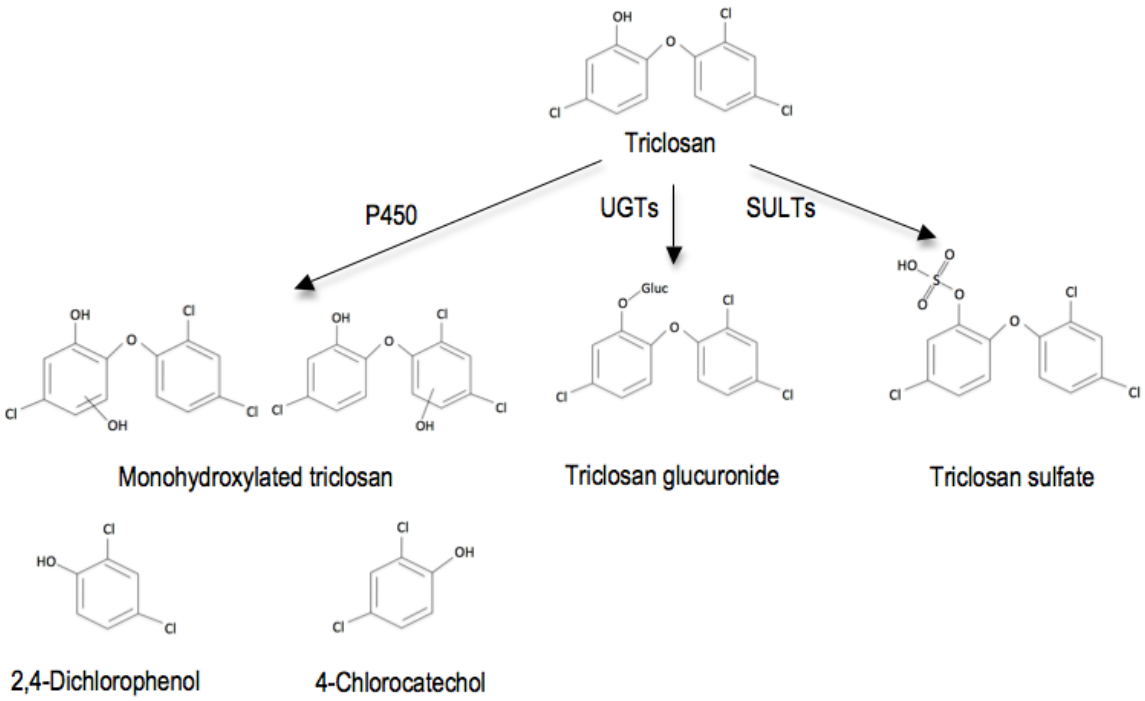



**Figure 1-6 Triclosan metabolism (figure obtained from Aguillon *et al*., 2010)**

### 1.3.3 Triclosan in Wastewater treatment plant influent and effluent

Triclosan is rinsed down the drain with normal usage into wastewater treatment plants (WWTP) alongside other commercial and domestic waste water. Concentrations of triclosan in wastewater influent as high as 22 µg $L^{-1}$ have been reported (Yu *et al.*, 2006) with concentrations vary due to season (Lindström, 2002; Waltman, 2006), between region and with the number of inhabitants in the catchment area (Nishi *et al.*, 2008). Up to 99% of triclosan can be removed from

sewage effluent during secondary sewage treatment (McAvoy *et al.,* 2002), resulting in reduced effluent concentrations ranging from 0.04 µg $L^{-1}$ (McAvoy *et al.,* 2002) to 2.5 µg $L^{-1}$ (Yu *et al.*, 2006). Concentrations also varying with the processing technology employed in the WWTP (Table 1-2). Removal rates are generally higher for activated sludge plants (Bester, 2005) than for trickling filter plants (McAvoy *et al*., 2002).

**Table 1-2 Triclosan concentrations in influent, effluent and the % removal efficiency in trickling filter and activated sludge treatment plants in Europe and North America.**

| Type of WWTP | Influent (µg $L^{-1}$) | Effluent (µg $L^{-1}$) | Removal (%) | Country | Author |
|---|---|---|---|---|---|
| **TF** | 8.0 | 2.5 | 69 | USA | Yu *et al* (2006) |
| **TF** | 0.78 – 1.62 | 0.01 – 0.06 | 92.3 – 99.4 | USA | McAvoy *et al* (2002) |
| **TF** | 3.8 – 16.6 | 1.6 – 2.7 | 57.9 – 87.3 | USA | Reiss *et al* (2002) |
| **TF** | 7.5 – 11.98 | 1.1 – 0.34 | 90.8 – 95.5 | UK | Reiss *et al* (2002) |
| **TF** | 3.7 | 0.13 | 96.5 | UK | Kanda (2003) |
| **AS** | 5.2-10.7 | 0.24-0.41 | 95.4-96.2 | USA | Reiss *et al* (2002) |
| **AS** | 7.51 – 21.9 | 1.1 – 0.47 | 93.7 – 95.0 | UK | Reiss *et al* (2002) |
| **AS** | 1.7 – 1.82 | 0.04 – 0.06 | 96.5 – 97.8 | USA | McAvoy *et al* (2002) |
| **AS** | 2.7 – 26.8 | 0.03 – 0.25 | 97.7 – 99.2 | USA | Waltman *et al* (2006) |
| **AS** | 21.9 | 1.1 | 95 | UK | Sabaliunas (2003) |
| **AS** | 1.2 | 0.051 | 95.8 | Germany | Bester (2003) |

**TF = Trickling filter AS = Activated sludge**

### 1.3.4 Triclosan in natural waters

Triclosan fate has been very well studied in the aquatic environment, and many studies have focussed on triclosan concentrations in sewage influent and rivers

receiving treated sewage effluent (Ellis, 2006; Kolpin *et al.,* 2002; Sabaliunas *et al.*, 2003; Morrall *et al.*, 2004; Waltman *et al.*, 2006; Halden & Paull, 2005). Concentrations differ regionally. Concentrations in receiving environments will depend on the ratio of emission to dilution and will vary greatly. A Swiss study reported concentrations ranging from 1.4-74 ng $L^{-1}$ in lakes (Lindstöm *et al.,* 2002), where as studies in the USA and Germany have reported triclosan concentrations in excess of 2300ng $L^{-1}$ and 5500 ng $L^{-1}$ respectively in streams (Koplin *et al*, 2002; Quintana and Reemtsma, 2004). Most triclosan levels are, however, more typically in the range of 4.1 – 4000ng $L^{-1}$ (Chau *et al.,* 2008; Moldovan, 2006; Kolpin *et al*., 2002; Lindström *et al*., 2002; Singer *et al*., 2002; Remberger *et al*., 2002; Zhang *et al*., 2007; Halden and Paull 2005; Chau *et al*., 2008; Coogan *et al*., 2007).

Fewer studies have reported triclosan in ground waters. Lindström *et al* (2002) detected triclosan in ground water at concentrations ranging from below the level of detection to approximately 0.4 ng $L^{-1}$, which is presented as evidence that some triclosan can percolate through the soil. This could derive, in part, from landfill leachate (Ellis, 2006). Despite the ban of disposal of biosolids to sea, triclosan is still detected at concentrations ranging from 0.001 – 6.87 ng $g^{-1}$ in the North Sea with a further 0.4 – 13 ng $g^{-1}$ in the solid particulate matter of the North Sea (Xie *et al*., 2008), which was attributed to high triclosan levels in the German river water feeding into the coastal waters. Other countries which are yet to ban sea disposal methods have detected triclosan at levels ranging from 1 ng $L^{-1}$ in the Charleston harbour, USA (DeLorenzo, 2008) to 28.9 – 110 ng $L^{-1}$ in the coastal waters of Hong Kong (Chau *et al.,* 2008; Wu *et al*., 2007).

#### 1.3.4.1 Degradation of Triclosan in aquatic environments

The biodegradation of triclosan in water is not fully understood. There appear to be several pathways depending on pH, UV exposure and the presence of other compounds in the water such as chlorine. Morrall *et al.,* (2004) used sodium bromide as a tracer to track a parcel of water from the source of triclosan emission in waste water effluent downstream in a river in Texas, USA. Four dissipation pathways were identified; photolysis, sorption, settling with sediment, and biodegradation.

##### *1.3.4.1.1 Photodegradation of aqueous triclosan*

One of the major removal pathways of triclosan in water is photodegradation (Lindström *et al.*, 2002). The preferred chemical reaction appears to be the replacement of a chlorine atom by a hydroxyl group (Ferrer *et al*., 2003), although, substitution of the chlorine atom by hydrogen and the cleavage of the C–O bond have also been observed (Sanchez-Prado *et al*., 2006). The latter pathway can lead to the production of 2,8-Dichlorodibenzo-p-dioxin (2,8-DCDD) (Latch *et al.,* 2003; Aranami and Readman, 2007). It has been observed that triclosan is less stable in seawater than in freshwater and that the dioxin is more stable in seawater than freshwater making dioxin production more likely as well as more persistent in the sea (Aranami and Readman, 2007), although dioxin formation accounted for only 1% of the degradation products. The formation of photodegradation products is dependent on pH. Triclosan is an ionisable compound with a dissociation constant (pKa) of 8.14 (Table 1-1). The pKa allows for the calculation of the proportion of

neutral and charged species at any pH (Figure 1-7). For triclosan, this means that in many environmental waters with high pH, a greater proportion will be in its anionic (phenolate) form than in the neutral form. The phenolate form is more abundant but is susceptible to photolysis resulting in the potential production of dioxins when exposed to sunlight or incineration (Latch *et al*, 2005). Furthermore, it is believed that only the neutral phenolic form of triclosan is toxic to aquatic organisms (Reiss *et al*, 2002) as molecules are less likely to cross the lipid membranes when in their ionised form.

In addition to dioxins, six other photo-products have been reported when waste water was exposed to UV light (Prado-Sanchez *et al.,* 2006). Two of the six products were present before exposure to UV light (monochlorophenol (MCP) and dichlorophenol (DCP)). Other products formed were dichlorohydroxydiphenyl, monochlorohydroxyphenol ether, 2,8-DCDD and a potential dioxin isomer (Figure 1-8). Both chlorophenol products have been reported to be toxic and are known for endocrine-disrupting activity (Canosa *et al.*, 2005).

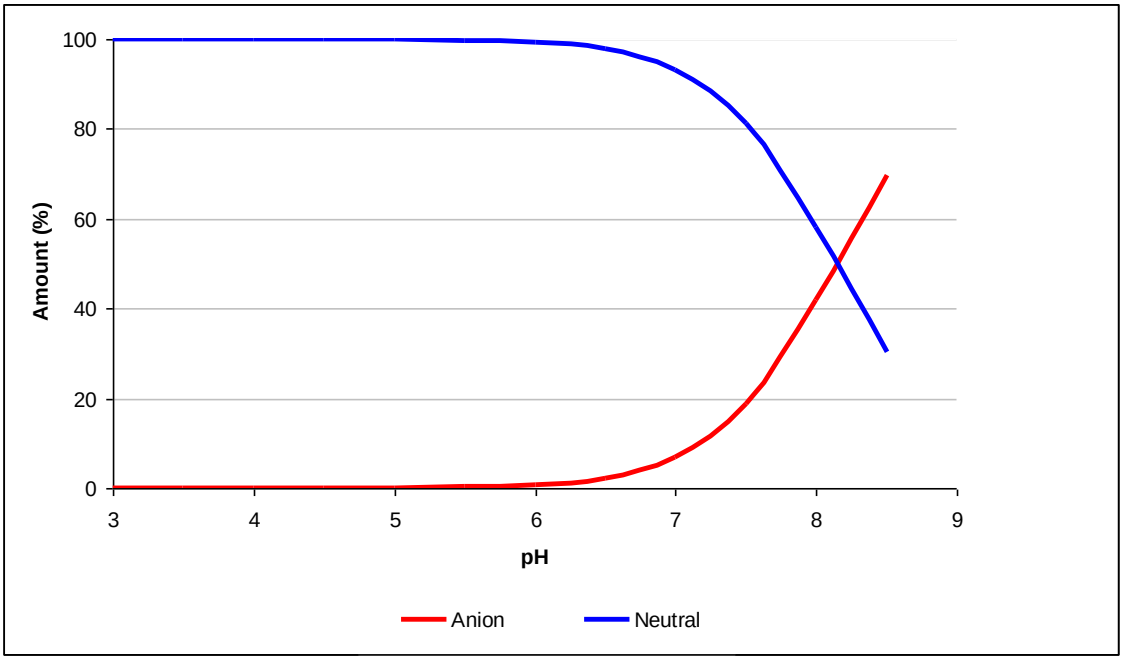



**Figure 1-7 A graphical representation of the ratio of anionic to neutral triclosan at various pH values, calculated using the equation used in Thomas and Foster, (2005).**

#### *1.3.4.1.2 Biodegradation and the formation of Methyl triclosan*

There are several ways in which triclosan can degrade as a consequence of biological action. In natural environmental matrices methyl triclosan (Me-TCS or 5-chloro-2-(2, 4-dichlorophenoxy) anisole) is the principal transformation product which has, thus far, been identified. Whilst triclosan is an antibacterial agent, Me-TCS does not possess antibacterial qualities and there are no known industrial applications. Compared to triclosan, Me-TCS is more lipophilic and environmentally persistent and it is not susceptible to photolysis (Chu and Metcalfe, 2007; Coogan *et al*., 2007), suggesting it has a high bioaccumulation potential in aquatic organisms (Glasser, 2004; Ying and Kookana, 2007). Me-TCS is known to form in WWTPs and is commonly detected in surface waters and sewage effluent (Halden and Paull, 2005;

Lindstrom *et al*., 2002; McAvoy *et al.,* 2002). It is thought to be formed by bacterial methylation of the parent triclosan under aerobic conditions in the sewage treatment plant (Boehmer *et al*., 2004; Balmer *et al*., 2004), in rivers (Kantiani *et al*., 2008) and in soil (Waria *et al*., 2011). Levels are much higher in wastewater effluent than influent (Lindström *et al*, 2002) and values as high as 1000 ng $L^{-1}$ have been reported (McAvoy *et al*, 2002). Thus far, to our knowledge there is only one report of Me-TCS being present in sewage sludge and in soils amended with sludge (Waria *et al*., 2011).

The concentration of Me-TCS in fish in four Swiss lakes was reported to be as high as 365 ng $g^{-1}$ (Lipid weight) in fish living in lakes recieving WWTP effluent, whereas Me-TCS could not be detected in fish from lakes which did not receive wastewater effluent (Balmer *et al,* 2004). Thus far, no toxicity studies have been published on Me-TCS.

#### *1.3.4.1.3 The action of chorine on triclosan*

When triclosan comes into contact with chlorine from water treatment or from bleach it is known to form various closans (Figure 1-8). Several triclosan closans have been identified in laboratory studies including Tetra–II (4,5-dichloro-2-(2-4-dichloro-phenoxy)-phenol), Tetra-III (5,6-dichloro-2-(2-4-dichloro-phenoxy)-phenol) and penta-closans (4,5,6-trichloro-2-(2-4-dichloro-phenoxy)-phenol) in excess hypochlorite (bleach) in water (Kanetoshi *et al.,* 1987; Onodera *et al.,* 1987). The closans have also been identified at low concentrations in sewage influent, effluent

and digested sludge (McAvoy *et al*., 2002). Triclosan has also been shown to form chloroform and other halomethanes during the chlorination of drinking water (Rule *et al*, 2005). These can be extremely toxic to humans and are considered as potential carcinogens as defined by the International Agency for Research on Cancer (IARC), although the levels produced are below the levels thought to be harmful.

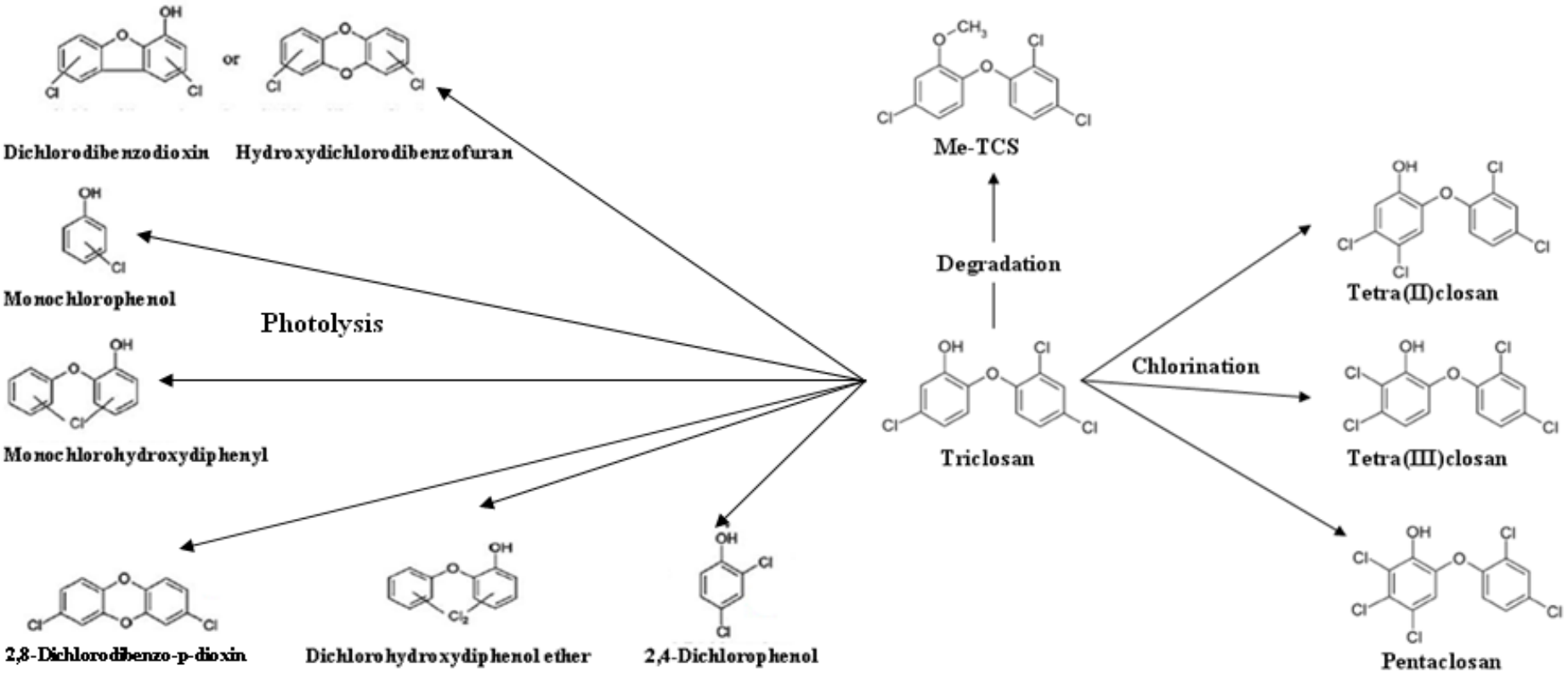



**Figure 1-8 The degradation products and pathways of triclosan in aquatic environments**

### 1.3.5 Triclosan toxicity to aquatic organisms

The majority of aquatic toxicity studies are carried out using standard protocols on 'standard' test organisms, such as *Daphnia magna* (survival, growth and reproduction) or algae (Wilson *et al.*, 2003). A 48-h median effective triclosan concentration ($EC_{50}$) for *Daphnia magna* of 390 µg $L^{-1}$ has been reported, with a no observed effect concentration (NOEC) of 200 µg $L^{-1}$ (Orvos *et al*., 2002). These concentrations are generally above the predicted environmental concentrations from most models which often estimate exposures in the range of 0.010 – 0.029 µg $L^{-1}$ (Bester, 2004; Boyd *et al.,* 2004) and even the most extreme concentrations of 5.5 µg $L^{-1}$ observed by Quintana and Reemtsma, (2004) and 8.5 µg $L^{-1}$ predicted by Capdevielle *et al,* (2008), were lower than the Daphnia NOEC.

Algae are generally believed to be extremely sensitive to triclosan. The algal species *scendedesmus subspicatus* has a 96-h $EC_{50}$ for growth of 1.4 µg $L^{-1}$ and a 96-h NOEC of 0.69 µg $L^{-1}$. This effect is believed to be algistatic rather than algicidal. When algae were transferred into a triclosan free medium, growth restarted almost immediately (Orvos *et al.,* 2002). The predicted no effect concentration (PNEC) for triclosan based on the algal NOEC data and following standard guidelines for hazard characterisation outlined in the TGD (EU, 2003) is 70 ng $L^{-1}$ (Capdeveille *et al*., 2008). A PNEC of 1550 ng $L^{-1}$ has been proposed by Capdeville *et al*, (2008) on the basis of the $5^{th}$ percentile potentially affected fraction in a species sensitivity distribution (SSD).

Aquatic animals are generally less sensitive to triclosan than algae. Rainbow trout have an $EC_{50}$ and a NOEC of 350 µg $L^{-1}$ and 34 µg $L^{-1}$, respectively (Ciba, 2001). However, Veldhoen *et al.* (2006) observed that American bullfrog tadpoles with growth media containing triclosan concentrations as low as 0.15 µg $L^{-1}$ for four days showed increased hind limb growth and decreased body weight development. Lower concentrations of 0.03 µg $L^{-1}$ still had an effect on tadpole development resulting in altered mRNA expression in the tail fin and brain as well as induced weight loss. Other studies have shown triclosan also has the potential to cause changes in fin lengths and fish sex ratios (Houtman *et al.,* 2007).

DeLorenzo *et al.* (2008) undertook toxicity tests on estuarine systems and identified that triclosan impaired phytoplankton growth and inhibited bacterial metabolism in simulated laboratory studies at high triclosan concentrations. It was also noted that grass shrimp bioaccumulated lethal levels of methyl triclosan. However, modelling to simulate river conditions in Europe and the United States has estimated that PEC to PNEC ratios are usually acceptable, suggesting risks to aquatic species are low even under the highest potential exposures that could occur after high triclosan emission episodes (Capdeville *et al*., 2007). This point was also made by Price et al. (2010) who predicted concentrations of triclosan in rivers using a spatially-resolved model that showed that the majority of the river reaches had concentrations lower than 70 ng $L^{-1}$ even under low flow conditions.

#### 1.3.5.1 Triclosan as a possible endocrine disrupter

Triclosan is thought to be weakly androgenic causing increased hermaphrodites and feminisation in male fish (Geara-Matta *et al*., 2011). Several studies have determined that triclosan has weak endocrine disrupting properties to various species. For example, the biomarker used to detect endocrine activity (vitellogenin induction), which is normally only detected in female organisms (Johnson and Sumpter, 2001), has been identified in the male clawed frog after exposure to triclosan (Ishibashi *et al*., 2004). Triclosan has also been observed to disrupt thyroid mediated development in the North American bullfrog (Veldhoen *et al*., 2006) affecting growth and development at environmentally relevant concentrations. Hinther *et al.* (2011) looked again at the disruption triclosan caused to tadpole tail fin development and found that in actual fact triclosan had no effect on thyroid action. However, methyl-triclosan did have significant effects on stress indicators as well as thyroid action suggesting that triclosan itself is less problematic than its metabolite.

### 1.3.6 Triclosan in biosolids

Due to the relatively low water solubility of triclosan (12mg $L^{-1}$ at 20˚C) and its high octanol/water partitioning coefficient (Log $K_{OW}$ of 4.8) at neutral pH (Reiss, 2002), it is very likely that a significant fraction of triclosan will sorb to sludge during sewage treatment (Halden and Paull, 2005). Most studies of WWTP process have looked at the removal of triclosan from the influent and until recently there were very few studies that considered the concentration of triclosan in sludge itself (Heidler and Halden, 2006).

Triclosan levels in sewage sludge and biosolids vary greatly with batch, type, country and season. Concentrations in digested sewage sludge have been reported to range from 0.4-8 mg $kg^{-1}$ and 0.5-15.6 mg $kg^{-1}$ (Bester, 2003; McAvoy *et al.,* 2002), up to 10 - 24.6 mg $kg^{-1}$ (Barron *et al*., 2008) and even 20-55 mg $kg^{-1}$ (Heidler and Halden, 2006). Triclosan levels measured in dried granular biosolids are also highly variable with reported levels of between 0.09 – 7 and 0.09-16.8 mg $kg^{-1}$ (Cha and Cupples, 2009; Ying and Kookana, 2007) up to 0.68-11.55 mg $kg^{-1}$ (Chu and Metcalfe, 2007) and as high as 3-33 mg $kg^{-1}$ (Kinney, 2006). Although concentrations are high in the biosolids, they are generally lower than in the digested sludge. The reduced concentration can possibly be explained by the heat process used in producing this dry pellet (450˚C), which can reduce triclosan concentrations, although not eliminate it completely. Often, before sewage sludge is applied to soil, it can be stored for a long period of time (days to months). During this time, triclosan has shown to be relatively stable within the sludge and is resilient to photo-degradation as well as biodegradation in both aerobic and anaerobic studies during this period (Chenxi *et al.,* 2008).

### 1.3.7 Triclosan fate in soil

The potential fate of triclosan in soil is illustrated schematically in Figure 1-9. The application of biosolids to land has been of concern for many years with regards to heavy metals (Alloway and Jackson, 1991) and nutrients (Withers *et al.,* 2001) and their potential for run-off into surface waters. More recently, veterinary pharmaceuticals have been measured in run-off from fields treated with animal

slurries (Davis *et al.,* 2006). This suggests that organic compounds present in sludge may be vulnerable to loss prompting investigations of the fate of compounds such as triclosan in soil (Topp *et al.,* 2008). In recent years there has been an increased focus on biosolid-borne organic pollutants such as surfactants, particularly LAS (Schramm *et al*., 1995; Ou *et al*., 1996: Carlsen *et al*., 2002; Schowanek *et al*., 2007; Kannan *et al.,* 2007), PAHs (Beck *et al*., 1996; Oleszczuk, 2006; Santos *et al*., 2007), flame retardands (Sun *et al*., 2008) and pharmaceuticals (Diaz-Cruz *et al*., 2003; Xu *et al*., 2009; McLellan and Halden, 2010). Despite increased interest, there have been relatively very few studies on the fate of home and personal care ingredients in soil after the application of biosolids and sewage sludge. This is in part, due to the complex matrices of soil and sludge (Heidler and Halden, 2006) and the difficulties of obtaining a representative, homogenous sample compared to water sampling.

There have been several controlled laboratory studies on triclosan fate but very few have been performed at field scale. Results obtained in the laboratory can give a good indication of how triclosan dissipates. However, laboratory experiments can overestimate actual degradation rates in the field (Al-Rajab *et al.,* 2009). For example, laboratory degradation half lives as low as 18 days (Ying *et al.,* 2007), whereas half lives obtained in field studies tend to be longer. A half life of 107.4 days was reported by Lozano *et al.* (2010) for example, and for methyl-triclosan the field half-life was 443 days (Lozano *et al*., 2012).

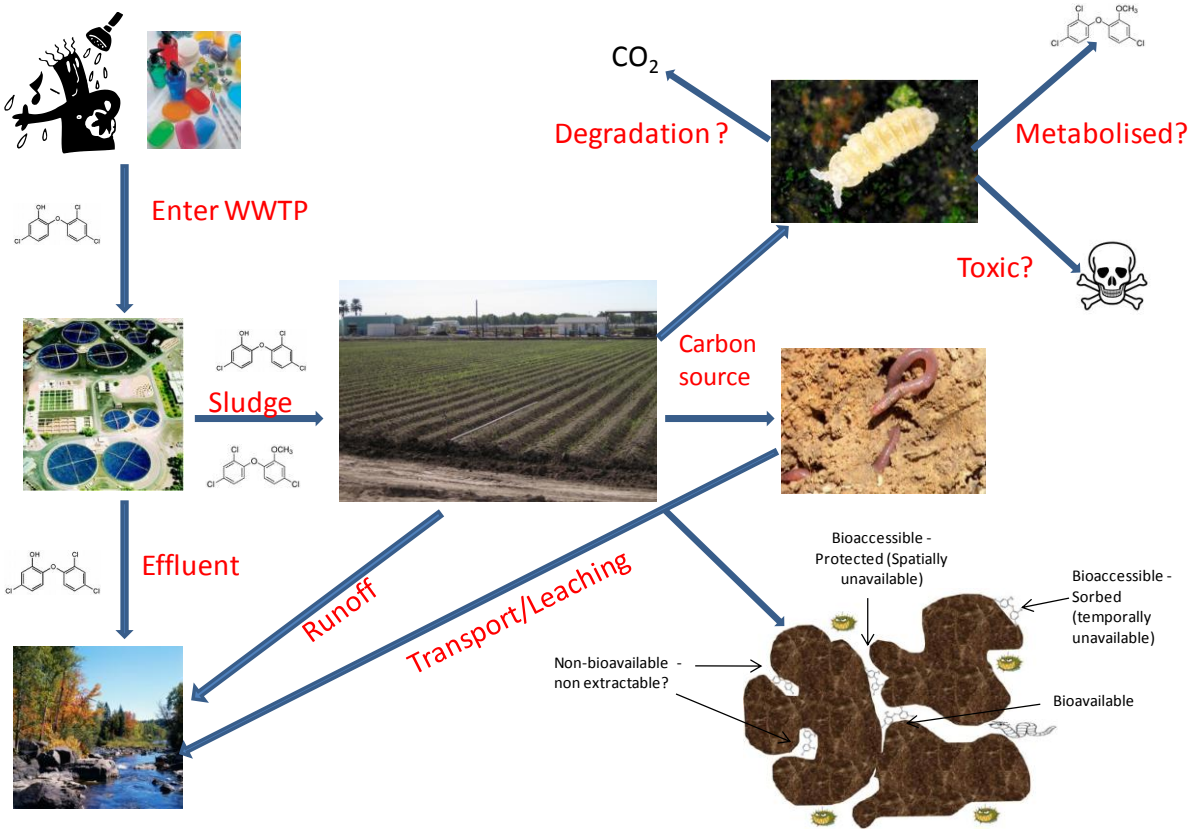



**Figure 1-9 the potential fate of triclosan from initial usage.**

A chemical is classified as persistent if its dissipation half-life exceeds 120 days (180 days - very persistent) in sediment and soil (Registration, Evaluation, Authorisation and restriction of Chemicals: REACH). Therefore, even the field half-life of 107 days, is not considered persistent, although triclosan is considered as very persistent in sediments (Wilson *et al*., 2008). Additionally, Langdon *et al.* (2012) could not estimate a degradation half-life as they saw no apparent loss at all in the field and a separate laboratory study where triclosan was applied to the soil through the addition of dried triclosan pellets produced a half-life of 301 days (Langdon *et al*., 2011), which exceeds the 180 day REACH persistence threshold. There is a need to obtain a better insight in to the mechanisms that could be responsible for the variation in

degradation half-lives reported. For example, temperature and soil moisture content could affect biological transformation, volatilisation, photodegradation, bioturbation, run-off and leaching.

#### 1.3.7.1 Volatilisation of triclosan from soil

Volatilisation can be a major removal pathway for some organic compounds present in soil. Propensity for volatilisation is a function of the vapour pressure, aqueous solubility and organic carbon to water partitioning coefficient ($K_{OC}$) of the chemical as well as soil properties and climatic influences such as temperature and soil water content. The method of applying biosolids to land can also affect the rate at which the compound is lost to the environment. Compounds present in biosolids which are surface applied to the soil have the potential for immediate volatilisation, whereas biosolids which are injected or incorporated below the soil surface will be less likely to be lost to the environment. High soil and biosolid organic matter content tends to increase sorption of the compound and therefore reduce volatilisation (Crompton, 2000). Triclosan has a low vapour pressure ($1.84 \times 10^{-4}$ Pa), a low Henry's law constant of $2.27 \times 10^{-4}$ Pa $m^3$ $mol^{-1}$ (Bock *et al*., 2010), a high octanol\water partitioning coefficient (log $K_{OW}$) of 4.76 and a high estimated octanol\air partition coefficient (log $K_{OA}$) of 11.45. These properties suggest that triclosan has a very low likelihood of volatilisation.

#### 1.3.7.2 Photodegradation of triclosan in soil

Many organic compounds including triclosan are known to degrade when exposed to solar radiation (Tixier, 2002). One of the major removal pathways of triclosan in water is photodegradation (Lindström *et al.*, 2002), which can lead to the production of 2,8-Dichlorodibenzo-p-dioxin (2,8-DCDD) (Latch *et al.,* 2003).

Photodecomposition of organic contaminants in biosolids has not been well studied. However, much of the contaminant will be absorbed within the soil matrix and, hence, largely protected from most wavelengths so the extent to which this process is important is unknown but unlikely to be high. There have been no reported studies of triclosan photodegradation in soils to the best of our knowledge.

#### 1.3.7.3 Triclosan movement through the soil

Triclosan can move in soil via leaching of dissolved phase compound, via leaching of colloids with associated compound, via diffusion through the water filled pore space and through bioturbation (the physical transloacation of soil solids by soil fauna such as earthworms). Leaching of an organic chemical is dependent on its partitioning in the soil matrix. For triclosan, the relatively high $K_{OC}$ value (Table 1-1) means that it will have a low pore water concentration, particularly when the organic carbon content is high as it is in sludge. However, the presence of solvents and surfactants in biosolids may act to increase mobility (Crompton, 2000). Soil properties such as clay content, pH and cation capacity may also be important in determining propensity to leach (Crompton, 2000). Depending on the soil pH, a compound may ionize. Ionization can make a chemical more polar and water-soluble, hence

increasing its mobility and bioavailability for degradation. Alternatively or it may remain in the non-ionized form where it is more likely to sorb to organic matter in the soil (MartÍ *et al*., 2011).

Rainfall, soil moisture content and temperature can also contribute to the tendency for movement of a chemical through soil. Rainfall intensity may generate overland flow. This overland flow can contain elevated concentrations of nutrients as well as organic pollutants if they are present on or near the soil surface, which can be transferred to rivers and streams. Although triclosan is likely to sorb considerably to soil and sludge particles, it may still be mobile in overland flow if there is particulate movement (erosion). Preferential or bypass flow, where water and solutes move down cracks bypassing the soil matrix, is a widely recognised pathway in some soils (e.g. Ogawa *et al*., 2000; Jianzhi *et al*., 2007; Morales *et al*., 2010) and may allow sorbed chemicals to be transported even where pore water concentrations are low.

Factors such as biosolid type (Edwards *et al.,* 2009; Lapen *et al.,* 2008) and biosolid application (Al-Rajab *et al.,* 2009; Topp *et al.,* 2008) can have significant effects on triclosan mobility. Liquid biosolids have been seen to increase the formation of large macropores in soil, which facilitates gravimetric flow of the aqueous phase through the soil, increasing the amount of triclosan found in tile drainage (Edwards *et al.,* 2009). These macropores will also reduce contact time with the soil matrix and hence tend to reduce degradation and transformation (Larsbo *et al.,* 2009). Once in ground water, or in the sub-soil, triclosan persistence may increase due to reduced

oxygen concentrations (Sabourin *et al.,* 2009) and due to the reduced number and diversity of micro organisms present. Dry biosolid pellets conversely do not have the same transport potential as the liquid slurry. Transport of pellets will depend on rainfall events and irrigation regime (Edwards *et al.,* 2009). Dry pellets generally have a longer contact time with the soil and increased sorption to the organic matter. Approximately 1% of triclosan found in dry sludge pellets was recovered in agricultural run-off when sludge was applied to land by Sabourin *et al.* (2009). However, when the same amount of triclosan was applied in a liquid biosolid, the fraction of triclosan detected in overland flow increased to 3% of that applied (Lapen *et al.,* 2008).

If sludge is surface-spread it will have a greater likelihood of being lost to water courses in overland flow than if it is incorporated. Levels as high as 258 ng triclosan $L^{-1}$ have been observed in agricultural runoff where sludge had been broadcast, after one day, compared to undetectable levels in soils where the sludge was incorporated (Topp *et al.,* 2008).

### 1.3.8 Triclosan toxicity in the terrestrial environment

#### 1.3.8.1 Toxicity to invertebrates

Toxicity studies on soil invertebrates are scarce and the only known toxicity tests performed on triclosan in the terrestrial environment have been on earthworms. The survival rates of adult earthworms (*Eisenia foetida)* were studied at various nominal

test concentrations between 64 -1026 mg $kg^{-1}$ (dry weight) by Wuethrich (1990). Mortality was low (within the acceptable range of 10%) even at the highest concentration giving a NOEC of 1026 mg $kg^{-1}$. However, no other chronic endpoints such as reproduction or growth were investigated, so sensitivity is unknown. Soil concentrations after sludge application are generally observed to be in the range of 39 µg $kg^{-1}$ (Langdon *et al*., 2012) to 98 µg $kg^{-1}$, and the concentration within biosolid aggregates have been reported to be as high as 1750 µg $kg^{-1}$ (Gottschall *et al.,* 2012). These concentrations would not be expected to be toxic to earthworms. However, triclosan bioaccumulation is possible. The ratio of triclosan concentration in earthworms to that in the soil was reported to be 10.8 on the first sample collection date and 27 on the second 140 days later, suggesting that bioaccumulation was occurring (Kinney *et al*., 2008).

#### 1.3.8.2 Toxicity to plants

Several studies have looked at triclosan toxicity to plants. Hoberg (1992) studied the phytotoxic effects of triclosan on various endpoints in six different plant species over 21 days. Corn and soybeans showed no adverse effects to triclosan exposure under the test conditions and tomatoes and wheat were only affected at the highest test concentration of 0.93 mg $kg^{-1}$. Cucumbers were most affected by triclosan with a NOEC of 0.096 mg $kg^{-1}$. Alternate toxicity tests on cucumber seedlings generated a NOEC of 1 mg $kg^{-1}$. Differences in cucumber sensitivity to triclosan are due in part to different soil organic carbon contents. Higher organic matter concentrations tend to reduce triclosan bioavailability to plants (Fuchsman *et al.,* 2010).

#### 1.3.8.3 Toxicity to microbes and microbially mediated processes

Since triclosan is an antimicrobial substance, it has the potential to exert toxicity in soil micro-organisms, This in turn could influence important microbially mediated processes such as decomposition and nitrification. Several studies have investigated triclosan effects on microbial function and diversity (e.g. Liu *et al*., 2009; Ying *et al*., 2007; Waller and Kookana, 2009). These are reviewed more comprehensively in Chapter 7.

## 1.4 Thesis structure

### 1.4.1 Aims and objectives

Although triclosan has been extensively studied in the aquatic environment and in the wastewater treatment process, there are still significant knowledge gaps on its fate and effects in soils. It is the intention of this thesis to the bridge some of these knowledge gaps and to address some of the outstanding uncertainties.

The aims and objections of the thesis are summarised in Figure 1-10. The overall aim is to determine the key factors affecting the degradation, mobility and effects on microbial community composition and function of triclosan in soil receiving sewage sludge. This thesis is divided into two parts. The first examines aspects of triclosan fate in soils amended with biosolids, including degradation, translocation and the

formation of non-extractable residues. The second explores some of the implications of triclosan addition for microbial community structure and specific functions. In this section of the thesis, the microbial respiration response to triclosan addition is examined in terms of resistance and resilience concepts and changes in the community structure were investigated using phospholipid fatty acid (PLFA) profiling.

### 1.4.2 Thesis outline

Three chapters (2, 3 and 4) focus on the fate of triclosan in soil, and three chapters (5, 6 and 7) look at effects (See Figure 1-10). All research chapters (2-7) are written in paper format. Chapters 5 and 6 have already been published in *Environmental Toxicology and Chemistry* and *Soil Biology and Biochemistry* respectively. Chapter 4 has been accepted by *Environmental Pollution*, and chapter 7 has also been accepted by *Chemosphere* (subject to revisions). Where appropriate the objectives of each piece of work are framed in terms of specific hypotheses, which are summarised in Figure 1-10.

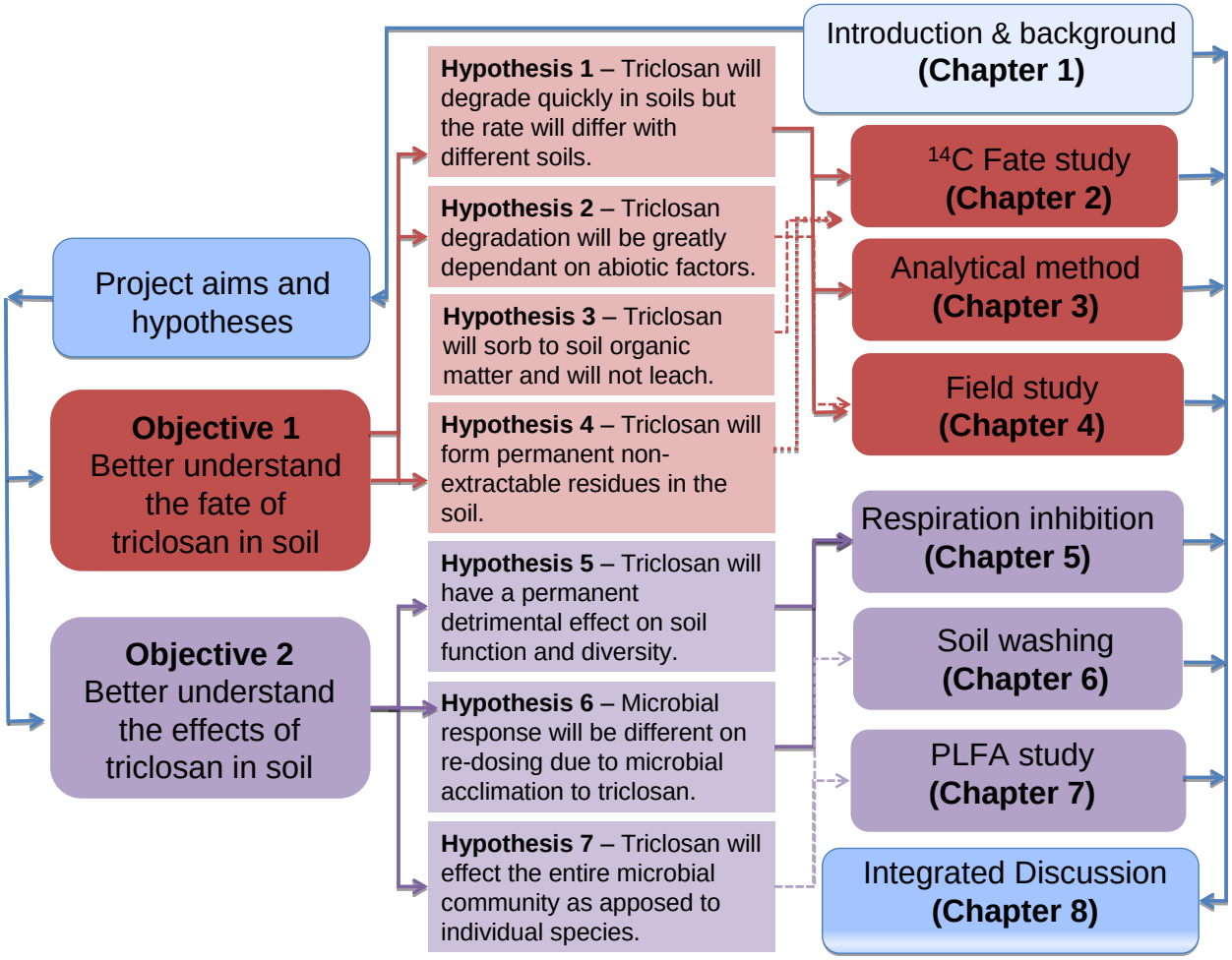



**Figure 1-10 Summary of thesis structure**

### 1.4.3 Chapter highlights

- Chapter one (current chapter), describes the context for the research including background information on the chemical itself and its fate in other environmental matrices. The thesis structure is described and aims and objectives laid out.

- Chapter two presents a laboratory degradation study using radio-labelled triclosan as a tracer. Specifically mineralisation, transformation and

degradation are examined including the formation of methyl triclosan. This chapter also looks at sequentially extracting triclosan from soils and the potential formation of bound residues.

- Chapter three presents the development of an analytical method to simultaneously detect and quantify trace amounts of triclosan and methyl triclosan present in soils and biosolids.

- Chapter four details a 13 month field trial looking at the fate and dissipation of triclosan in three different soils amended with biosolids. In Chapter two, there was strong evidence that a significant fraction of triclosan degrades to form methyl-triclosan in soil. This chapter examines methyl-triclosan formation in the field for the first time.

- Chapter five investigates the effects of triclosan on basal and substrate-induced respiration and the respiration inhibition response of re-dosing.

- Chapter six documents the development of a soil washing procedure required to remove excess triclosan from soil samples before they can be analysed for their phospholipid fatty acid (PLFA) profiles without altering the microbial composition of the soil. Triclosan was found to co-elute with the fatty acid methyl esters on the gas chromatogram, masking the profile and any subtle changes in the phenotypic structure.

- Chapter seven examines whether changes in microbial community structure are induced by triclosan addition in the same experiment as that described in Chapter five. Phenotypic profiling was performed using PLFA analysis.

- Chapter eight attempts to summarise the key findings of the thesis and to highlight the implications of the findings for the environmental risk assessment of triclosan.

# Chapter 2

## The Fate of $^{14}$C-labelled triclosan in biosolid amended soils

## 2.1 Abstract

Triclosan is an anti-microbial substance used in a range of home and personal care products. It is efficiently removed from waste water in sewage treatment plants. However, a significant fraction is sorbed to sludge and can enter the terrestrial environment if sludge is applied to land. A laboratory mineralisation study of $^{14}$C-labelled triclosan was conducted in three soils (a sandy loam, a loamy sand and a clay soil). Triclosan was dosed directly on to the soil or dosed to biosolids which were then applied to the soil. A low rate of mineralisation was observed in all treatments with less than 7% mineralised after 6 months. Soils were extracted sequentially with $CaCl_2$, methanol, acetonitrile and hexane after 6 months of incubation. In general, only between 16 and 37% of the applied $^{14}$C was extractable. Radio-HPLC revealed that 7-15% of the originally applied $^{14}$C material that was extractable from the soils was present as parent triclosan with a greater fraction present as methyl triclosan (18-26%). This is lower than that observed in field soils where after a 12 month period between 39 and 66% of triclosan applied in biosolids was extracted in its methylated form (Chapter 4). In sterile soils, almost all of the original activity extracted, was present as parent triclosan with no methyl-triclosan present. The results suggest that degradation half-lives of triclosan in laboratory soils are higher when the triclosan is applied in biosolids than when the triclosan is applied directly. They also suggest that the fate of triclosan is affected by the formation of non-extractable residues. The formation of methyl triclosan, suggests that biodegradation is an important fate process.

## 2.2 Introduction

Triclosan (5-Chloro-2-[2, 4-dichloro-phenoxy]-phenol) is an antimicrobial agent which shows broad spectrum activity against gram-positive and gram-negative bacteria, moulds and yeasts (McAvoy *et al*., 2002). It is used in many personal care products such as shampoo, toothpaste and skin-care treatments, as well as in household products such as chopping boards and food containers in the form of “microban”. The concentration of triclosan in these products is approximately 0.1 - 0.3% (w/w). With normal usage, triclosan is rinsed down the drain and into sewage treatment plants. The fate of triclosan in sewage treatment has been well studied. In general, there is high removal with 95-99% of influent concentration being removed in well run activated sludge plants (Chau *et al*., 2008; Bester, 2003; McAvoy *et al*., 2002). Of this removal approximately 30% is believed to sorb to sludge since triclosan is relatively hydrophobic with a log octanol: water partition coefficient (log $K_{OW}$) of 4.8 (Reiss *et al*., 2002), suggesting that it will sorb appreciably to organic matter in sewage sludge (Heidler *et al*, 2006; Ying and Kookana, 2007; Kinney *et al*., 2008). The association of sludge to land can therefore represent a significant route for soil exposure to triclosan (Heidler, et al., 2006). About 10% of triclosan entering wastewater treatment plants is bio-transformed to produce the methylated metabolite - methyl triclosan, the remainder is presumed to be degraded into unknown metabolites (Bester, 2003; Heidler & Halden, 2007).

The fate and effects of triclosan released into aquatic environments has also been well studied (e.g. Singer *et al.*, 2002; Reiss *et al*., 2002; Lindstrom *et al.*, 2002;

Capdevielle *et al*., 2008; Wilson *et al*., 2008). However, thus far, very few studies have examined the fate of triclosan in soil. Degradation half-lives of triclosan have been reported to vary significantly between lab studies (e.g.18 days, Ying *et al*., 2007) and field studies (e.g.107.4 days, Lozano *et al*., 2010). Triclosan is not readily degradable using standardised screening tests such as OECD 301C and 302C (OECD, 1992), although triclosan can be readily degraded after microbial acclimation (Federle *et al.*, 2002). Triclosan is considered to be inherently degradable in aerobic soil but persistent in anaerobic soils (Wu *et al*., 2009; Ying *et al*., 2007). An early mineralisation study of triclosan applied to soil suggested a mineralisation half-life of 577 days (Ciba, 1994).

One potential reason for the lack of degradation reported in earlier studies could be the toxicity of triclosan to soil microbes at the relatively high concentrations required in these tests. Offhaus *et al* (1978), showed that triclosan concentrations as low as 2 mg $L^{-1}$ inhibited microbial breakdown of peptone molecules in sewage sludge. Triclosan concentrations in digested sludge are typically in the range of 10 to 24.6 mg $kg^{-1}$ in (Barron *et al*., 2008), although levels as high as 55 mg $kg^{-1}$ have been reported (Heidler and Halden, 2007).

It is important to better understand the factors which affect the degradation of triclosan such as soil physical and chemical properties (which can affect triclosan sorption, mobility and bioavailability) and the method by which triclosan is introduced

to the soil (e.g. liquid sludge has been shown to increase triclosan mineralisation, whereas the application of dry pellets was observed to reduce it compared to direct application of liquid triclosan in a solvent to soil: Al-Rajab *et al*., 2009). The aim of this study was to explore some of these controlling factors on the fate of triclosan in different soils, particularly mineralisation. $^{14}$C-labelled triclosan was employed in laboratory incubation experiments in three different soil types. Triclosan was introduced into the soil in different ways to determine whether this affected mineralisation. Soils were also extracted after 180 days to determine the form of the remaining radiolabel.

## 2.3 Materials and methods

### 2.3.1 Biometer flasks

The experimental method was adapted from the OECD 304A (OECD, 1981). Biometer flasks used in this study (Figure 2-1) were fitted with a side arm 4cm in length which bends down at a 90˚ angle for a further 2 cm connected to a scintillation vial. The arm is fully air tight and the use of these flasks in biodegradation studies has been previously validated by Haigh (1993).

**Figure 2-1** An example of the biometer flask set-up showing scintillation vials containing potassium hydroxide attached to the side arm of each flask.

### 2.3.2 Soil preparation

Samples of three different soils (a sandy loam, a clay and a loamy sand) were collected from Silsoe Farm in Bedfordshire, UK (26˚25'W, 52˚26'N; 25˚56'W, 52˚12'N and 26˚27'W, 52˚33'N respectively). All samples were taken from the upper 20 cm of agricultural fields which have been under an arable rotation dominated by winter wheat and peas for over 20 years. None of the sites had previously received biosolids.

Samples were refrigerated at 5 °C immediately after collection and transported to the laboratory where they were air-dried and sieved to ≤ 2mm. Aliquots of each soil (50

g) were weighed into biometer flasks and three treatments were established in triplicate: (i) sterilised soil receiving triclosan in ethanol, (ii) unsterilised soil receiving triclosan in ethanol and (iii) unsterilised soil receiving biosolids spiked with triclosan. There were 27 biometer flasks in total, nine for each soil type. Biosolids (dewatered and dried pellets) were kindly provided by a leading UK water company. The soil moisture content was adjusted to 60% maximum water holding capacity and the flasks were incubated at 20˚C for two weeks prior to commencing the experiment to allow the microbial biomass to settle down post sampling (Ritz *et al*., 2006). The $^{14}$C triclosan working solution had a concentration of 100 µg triclosan mL$^{-1}$ in ethanol and an activity of 37 kBq mL$^{-1}$. This was stored in the freezer at -20˚C when not in use.

### 2.3.3 Triclosan dosing

On day zero of the experiment, 0.5 ml of the working solution was pipetted on to the soil to give a nominal triclosan concentration of 1 mg kg$^{-1}$, with an activity of 0.37 kBq g$^{-1}$ dry mass. The soils were stirred and incubated at 20˚C. The sterile soils were spiked in the same way and to the same concentration after being autoclaved three times at 126˚C for 20 minutes. For the treatments receiving biosolids, aliquots of 1.5g of biosolid (dry pellets), were weighed into metallic weighing boats, and 0.5 mL of the working $^{14}$C-triclosan solution was added and thoroughly mixed by hand using a stainless steel spatula. After allowing the excess ethanol to evaporate from the biosolids for 10 minutes, the biosolids were added to each respective flask containing the soil. The weighing boats were then rinsed with 0.5 mL ethanol which was added to the flasks to minimise loss of radioactivity from the system. The

biosolids were manually mixed with the soil and incubated at 20°C as above. Immediately after dosing, a scintillation vial containing 4 mL of 0.1 mol potassium hydroxide (KOH) was attached to each flask to prevent loss of radioactivity.

### 2.3.4 Sampling

On each sampling occasion (between 1 and 180 days), the scintillation vial was removed and immediately replaced with another containing 4 mL 0.1 M KOH. 12mL of Hionic Fluor scintillation counting fluid was added to each sampled vial and activity was counted using a Packard Tri-Carb (Perkin Elmer) scintillation counter. Disintegrations per minute (DPM) were automatically corrected for quench.

### 2.3.5 Combustion of samples to estimate remaining radioactivity

At the end of the experiment (180 days), approximately 0.2g of each soil sample was weighed into a Combusto-Cone (Perkin Elmer) and placed onto the ignition basket of a sample oxidiser (Perkin Elmer 307 sample oxidiser). The timer was set for 4 minutes. As the sample is combusted, $^{14}CO_2$ is absorbed onto Carbo-sorb E (Perkin Elmer) and mixed with 12 mL of Permafluor E scintillation cocktail (Perkin Elmer). This was performed in triplicate for each sample. Samples were then counted using a Tri-carb scintillation counter as previously described. Control $^{14}$C reference material was also combusted and counted regularly to ensure complete combustion was occurring.

### 2.3.6 Sequential extraction of remaining $^{14}$C from soil

The remaining soil in each flask was removed and weighed into a 100 mL bottle with lid. Each bottle was shaken on a side to side shaker for 24 hours after the addition of 20 mL of 0.01M calcium chloride. Samples were then centrifuged at 2000 RPM for 20 minutes. The supernatant was evaporated under a gentle stream of nitrogen and reconstituted in 1 mL tetrahydrofuran (THF) ready for HPLC analysis. This step was repeated using methanol (MeOH), then acetonitrile (ACN), and finally with hexane (Hex).

### 2.3.7 Radio-High Performance-Liquid Chromatography

Analytes were resolved using an Agilent 1100 (Agilent Technologies) HPLC unit fitted with a Beta-RAM radio-detector (Lablogic Systems, Sheffield). The column used was a Prodigy C-18, 250 mm x 4.6 mm, 5 mm pore size (Phenomenex LTD, Cheshire UK ). The mobile phase used was 0.1% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B) at an initial ratio of 95% solvent A: 5% solvent B. This was ramped up to 100% solvent B over a 20 minute period and held at this ratio for 7 minutes before reverting back to 95% solvent A and 5% solvent B and holding for a further 8 minutes, resulting in a 37 minute run per sample. Under these conditions, triclosan eluted at 19 minutes and methyl-triclosan at 22 minutes. Control samples of $^{14}$C-labelled triclosan and $^{14}$C-labelled methyl-triclosan were also run

after every 20 samples, as were blank THF samples to verify that there was no triclosan carry over.

### 2.3.8 Extractability of triclosan over time

In order to determine the efficiency of the extraction method, how fast triclosan metabolites are formed and how quickly triclosan becomes un-extractable from the soil, 50 g of the sandy loam soil (dry mass) was directly spiked with 0.37 kBq $^{14}$C-triclosan $g^{-1}$ (1 mg $kg^{-1}$) soil as before and sequentially extracted following the extraction method above. Soils were destructively sampled in triplicate on Days 1, 7,14, 21, 28, 35 and 42 following spiking with $^{14}$C triclosan. The extracted and concentrated samples were reconstituted in 1 mL THF and analysed using radio-HPLC.

## 2.4 Results

### 2.4.1 Triclosan mineralisation

The mean fraction of applied radioactivity captured as $^{14}CO_2$ in each treatment over the course of the experiment is shown in Figure 2.2. Simple first order kinetics did not provide a good fit to the data. In all treatments there was an initial and short lived rapid efflux of $^{14}CO_2$ (to about 20 days), followed by a slower rate of $^{14}CO_2$ production. This pattern could be better described using a two-pool first order model of the form:

$$C = C_1 \exp(-k_1 t) + C_2 \exp(-k_2 t)$$

where $C$ is the concentration of radio-label remaining unmineralised (and, for the sake of the model, assumed to be untransformed) at time $t$, $C_1$ is the initial concentration of the labile pool, $C_2$ is the initial concentration of the recalcitrant pool and $k_1$ and $k_2$ are first order rate constants for the labile and recalcitrant pools, respectively. Cumulative $^{14}CO_2$ emitted was calculated as (1 - $C$).

Best fit model parameters are shown in Table 2-1 and best-fit curves are also shown in Figure 2.2. The labile pool had the shortest half-life for the direct spiking treatment and the longest half-life for sludge application. Half-lives for the recalcitrant pool were also much shorter in the treatments in which triclosan was applied directly, rather than with sludge.

The initial rate of mineralisation was most rapid in the case of the loamy sand soil compared with the other two soils but slowed down after about 20 days. Overall, the cumulative mineralisation was greatest in the clay soil. However, visual inspection of the data suggests that rates of mineralisation beyond about 70 days were similar in all three soils. In contrast, it was observed that when $^{14}$C triclosan was applied to soil via sewage sludge, the pattern of mineralisation in all three soils is very similar. This suggested that the mineralisation rate is relatively unaffected by soil properties and maybe largely controlled by interactions with the sludge matrix.

The fraction of activity which was trapped in the potassium hydroxide was always less than 1% in the case of the sterilised treatments and was similar for all three soils. The fact that the $^{14}CO_2$ appears to have been emitted from these soils suggests that they may not have been effectively sterilised or that there was some microbial contamination in the vessels.

**Table 2-1 Best fit parameters for the two-pool model (From equation above)**

| | Direct spiking | | | Spiking in sludge | | | Sterile soils | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sand | Loam | Clay | Sand | Loam | Clay | Sand | Loam | Clay |
| $K_1$ | 0.077 | 0.028 | 0.038 | 0.02 | 0.02 | 0.02 | 0.03 | 0.06 | 0.02 |
| $K_2$ | 0.002 | 0.003 | 0.004 | $9.4x10^{-7}$ | $1x10^{-7}$ | $7.8x10^{-6}$ | $4.3x10^{-7}$ | $1.4x10^{-6}$ | $3.2x10^{-7}$ |
| $C_1$ | 1.98 | 1.99 | 2 | 2.1 | 2.13 | 1.99 | 0.81 | 0.55 | 0.65 |
| $C_2$ | 98.02 | 98.01 | 98 | 97.9 | 97.87 | 98.01 | 99.19 | 99.45 | 99.35 |
| $t½_1$(days) | 9.03 | 25 | 18 | 39.23 | 37.87 | 38.07 | 22.8 | 11.2 | 29.1 |
| $t½_2$ (years) | 8.09 | 6.08 | 8.47 | 2011 | 18970 | 244 | 4452 | 1351 | 5959 |

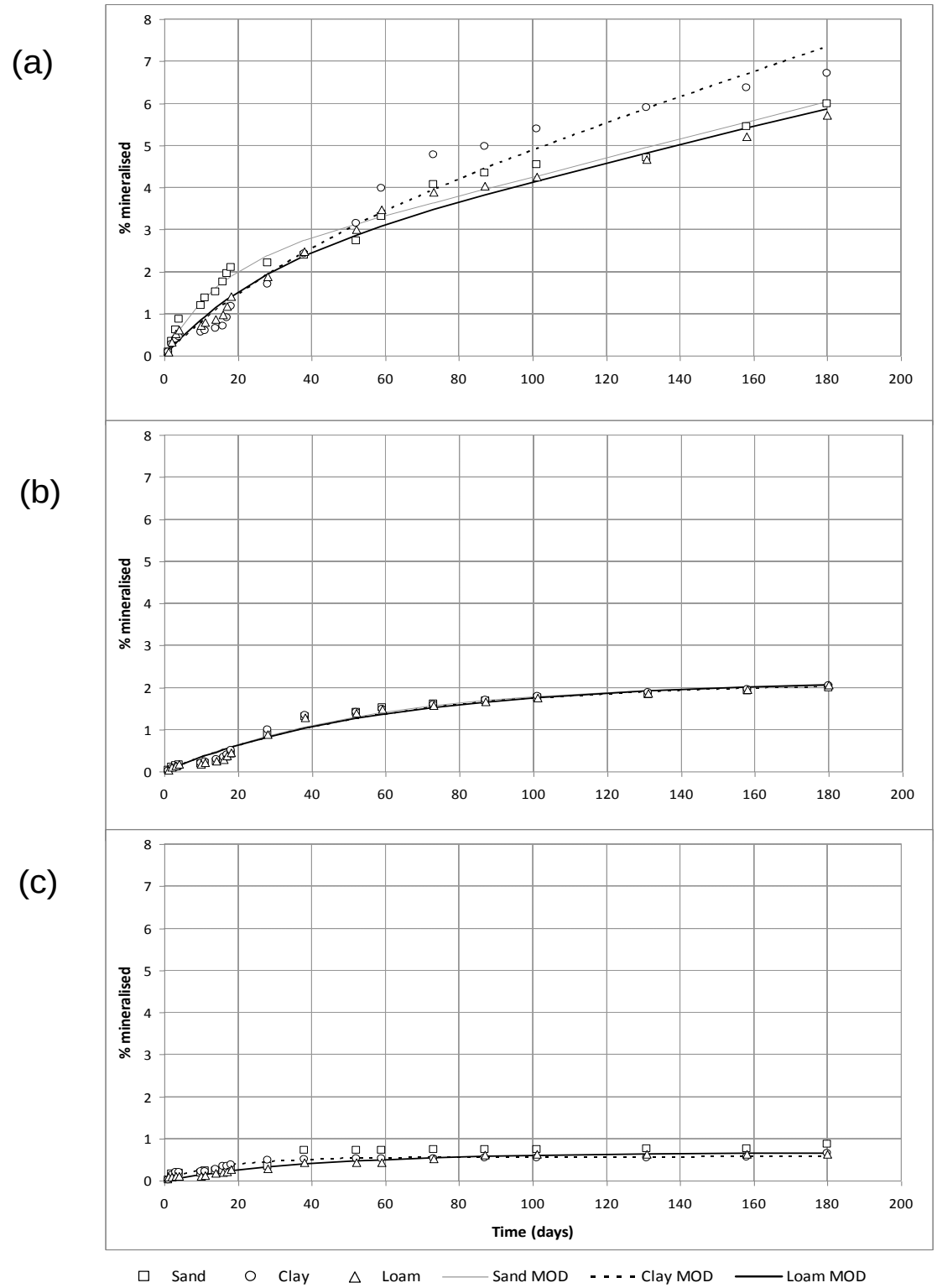



**Figure 2-2 The mean mineralisation of $^{14}$C labelled triclosan in the loamy sand, clay and sandy loam soils after a) direct application to the soils, b) application via sludge and c) direct application into sterile soils. Sand MOD, clay MOD and loam MOD refer to the 2-pool model fitted to the data.**

### 2.4.2 Activity mass balance

An estimation of total radiation mass balance was attempted in order to assess any unaccounted-for losses from the system. In each treatment, the cumulative quantity of radioactivity detected as $^{14}CO_2$ in each of the replicate three aliquots was combined. This was then added to the total radioactivity remaining in the biometer flask and compared with the radioactivity added to the system. The difference between the $^{14}$C spiked into the soil initially and the accounted-for radiation, is an unexplained loss. It is highly unlikely that any loss was due to volatilisation from the system as triclosan is non-volatile. Losses could be due to residual triclosan remaining on the weighing boat or in the pipette. The loss of radioactivity from the system was between 2 and 10 percent in all samples (Table 2-2).

**Table 2-2 Summary of the percentage of $^{14}$C mineralised, $^{14}$C recovery (combusted), and unaccounted for activity at the end of the experiment.**

| Treatment | Mineralised | Combusted | Unaccounted for |
|---|---|---|---|
| | Mean % | Mean % | Mean % |
| Loamy sand | 5.93 | 83.91 | 10.15 |
| Clay | 5.33 | 89.08 | 5.59 |
| Sandy loam | 5.02 | 89.56 | 5.42 |
| Loamy sand & Sludge | 1.98 | 90.94 | 7.08 |
| Clay & Sludge | 2.03 | 93.82 | 4.15 |
| Sandy loam & Sludge | 1.98 | 92.24 | 5.79 |
| Loamy sand sterile | 0.75 | 92.40 | 6.85 |
| Clay sterile | 0.54 | 97.70 | 1.76 |
| Sandy loam sterile | 0.63 | 95.99 | 3.38 |

### 2.4.3 Triclosan extraction

The different solvents used extracted triclosan to different degrees. Neither the 0.01 M $CaCl_2$ nor the methanol extracted any detectable triclosan from the soil after 6 months in all samples except for those from the sterilised clay and loamy sand soils and the unsterilised loamy sand soil and sandy loam receiving direct triclosan spiking. Methanol also extracted small quantities of activity from some of the sludge amended soils. Overall, extraction ranged between 16-37% of the originally applied activity. This is very low considering that more than 90% of the applied triclosan was still in most of the soils after 180 days (Table 2.2). The majority of triclosan was extracted using acetonitrile; however, hexane also proved to be effective. Slightly

more activity was extracted from the sludge applied treatments than from the treatments which were dosed with triclosan directly.

**Table 2-3 Solvent extraction of activity from soils after 180 days**

| Sample | Percent extracted with each solvent (%) | | | | Total % |
|---|---|---|---|---|---|
| | **$CaCl_2$** | **MeOH** | **ACN** | **Hex** | |
| Loamy sand | 1.26 | 1.73 | 8.02 | 5.31 | 16.19 |
| Clay | 0.00 | 0.00 | 23.67 | 10.25 | 33.92 |
| Sandy loam | 0.67 | 0.00 | 17.75 | 10.91 | 29.33 |
| Loamy sand & Sludge | 0.00 | 0.56 | 17.27 | 12.72 | 30.55 |
| Clay & Sludge | 0.00 | 0.41 | 22.73 | 13.37 | 36.5 |
| Sandy loam & Sludge | 0.00 | 0.00 | 19.14 | 14.68 | 33.82 |
| Loamy sand sterile | 0.00 | 0.58 | 18.27 | 10.84 | 26.26 |
| Clay sterile | 0.43 | 0.00 | 19.12 | 7.37 | 26.49 |
| Sandy loam sterile | 0.00 | 0.00 | 18.27 | 7.54 | 25.81 |

Radio-HPLC performed on the extracts showed the fraction of applied $^{14}$C extracted as triclosan, methyl triclosan and as unidentifiable material. Between 7-15% of the applied activity was recovered as triclosan and 18-26% as methyl-triclosan. The lowest amount of triclosan extracted was from the loamy sand soil. A relatively small proportion (0-3%) of the $^{14}$C was present in most of the samples in an unknown form (Figure 2-3).

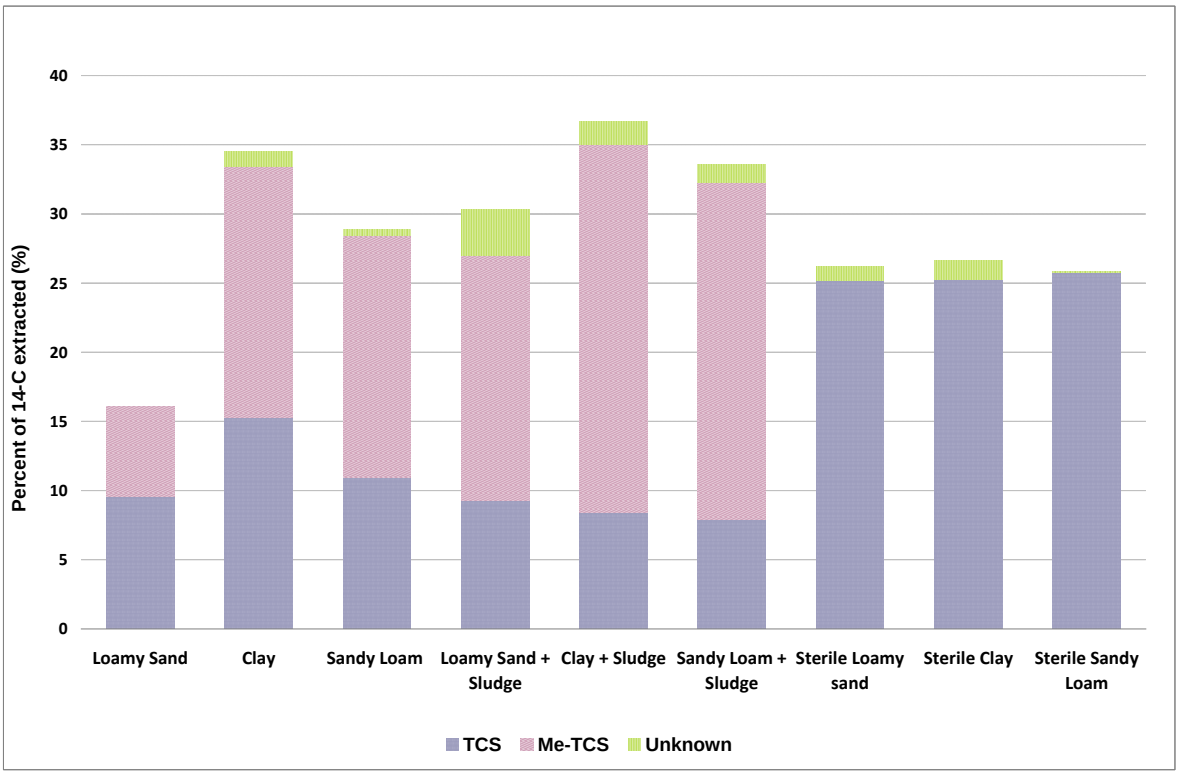



**Figure 2-3 The mean percentage of originally applied $^{14}$C extracted from each treatment broken down by the quantity found in parent triclosan form (TCS), a methyl-triclosan (Me-TCS) and in unidentified form.**

In the sterile soils, no methyl triclosan was extracted. Most extracted activity was still in triclosan form (>25% of originally applied $^{14}$C), although there were still traces of an unknown $^{14}$C compound.

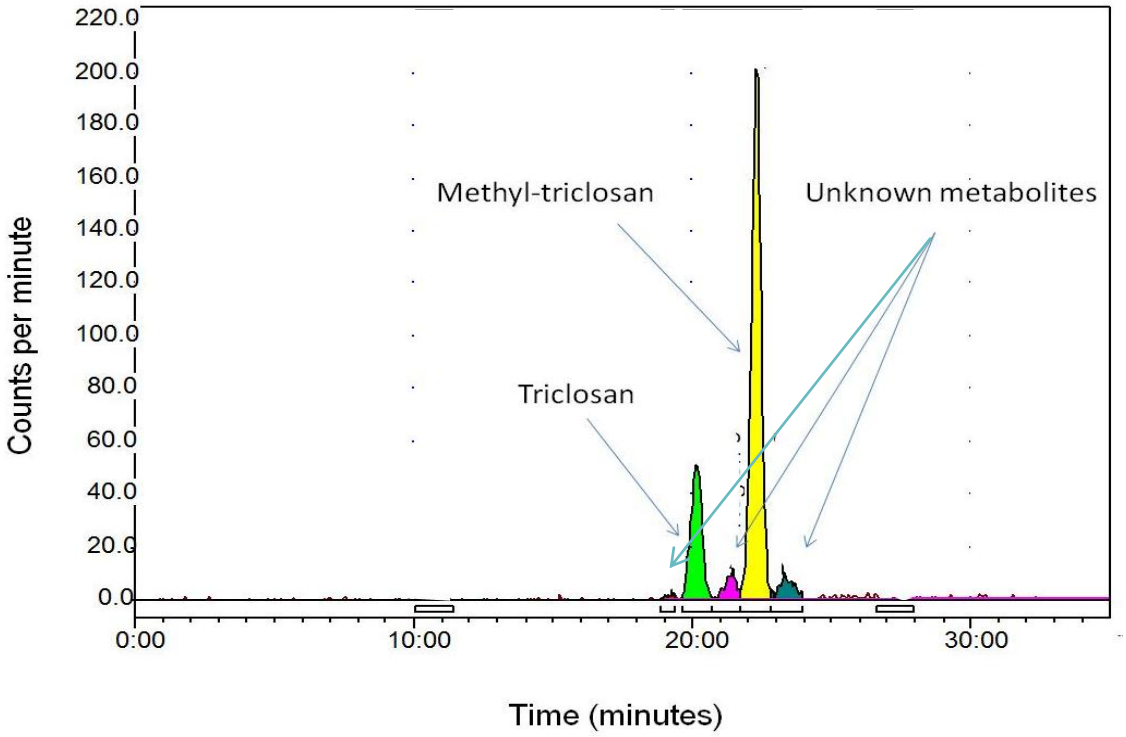



**Figure 2-4 A typical radio-HPLC chromatograph of an extract from the sandy loam soil spiked with sewage sludge dosed with $^{14}$C triclosan.**

The lowest concentration of parent triclosan and the highest concentration of methyl-triclosan and unknown metabolites were extracted from soils receiving sewage sludge. The majority of the unknown metabolites extracted were eluted from the HPLC column at a later time than triclosan which suggests that they are more hydrophobic than triclosan, since compounds are retained on this type of column based on their polarity (Figure 2.4).

### 2.4.4 The effect of soil type

In the soils directly dosed with triclosan, there was initially more mineralisation in the loamy sand soil, followed by the sandy loam and, finally, the clay soil. However, by Day 40 of the experiment, the clay soil had the highest cumulative mineralisation followed by the sandy loam and the loamy sand soil (Figure 2.2). At Day 20 there had been about 2% mineralisation in the loamy sand soil compared to 1.5% in the sandy loam soil and 1.15% in the clay soil. By day 100, on the other hand, there had been about 4.5% mineralisation in the loamy sand soil, 4.25% in the loam and 5.5% in the clay soil. By the end of the study, total mineralisation ranged between 5.7 and 6.7%. Over the whole course of the experiment, therefore, soil type had relatively little effect on triclosan mineralisation. There appeared to be no correlation between soil type and extractability or degradation of triclosan, although less methyl-triclosan was extracted from the directly dosed loamy sand soil, compared to the other soils (Figure 2.3).

### 2.4.5 Extraction of $^{14}$C triclosan over time

An attempt was made to determine the rate of methyl-triclosan formation by extracting dosed loamy sand soil at 1, 7, 14, 21, 28, 35, and 42 days. The day after adding the triclosan >95% of radioactivity could still be extracted from the soil. As time progressed, the fraction of remaining activity which could be extracted decreased progressively (Figure 2.5). After 28 days only 50% of the applied $^{14}$C could be extracted from the soil. Methyl-triclosan was detected from Day 14 (Figure 2.5) and methyl-triclosan formation increased progressively as triclosan

concentration decreased. Unknown triclosan metabolites were identified from Day 21 and abundance increased by Day 42. By the end of the study (Day 42), of the 45% of remaining activity, only 20% was present in triclosan form.

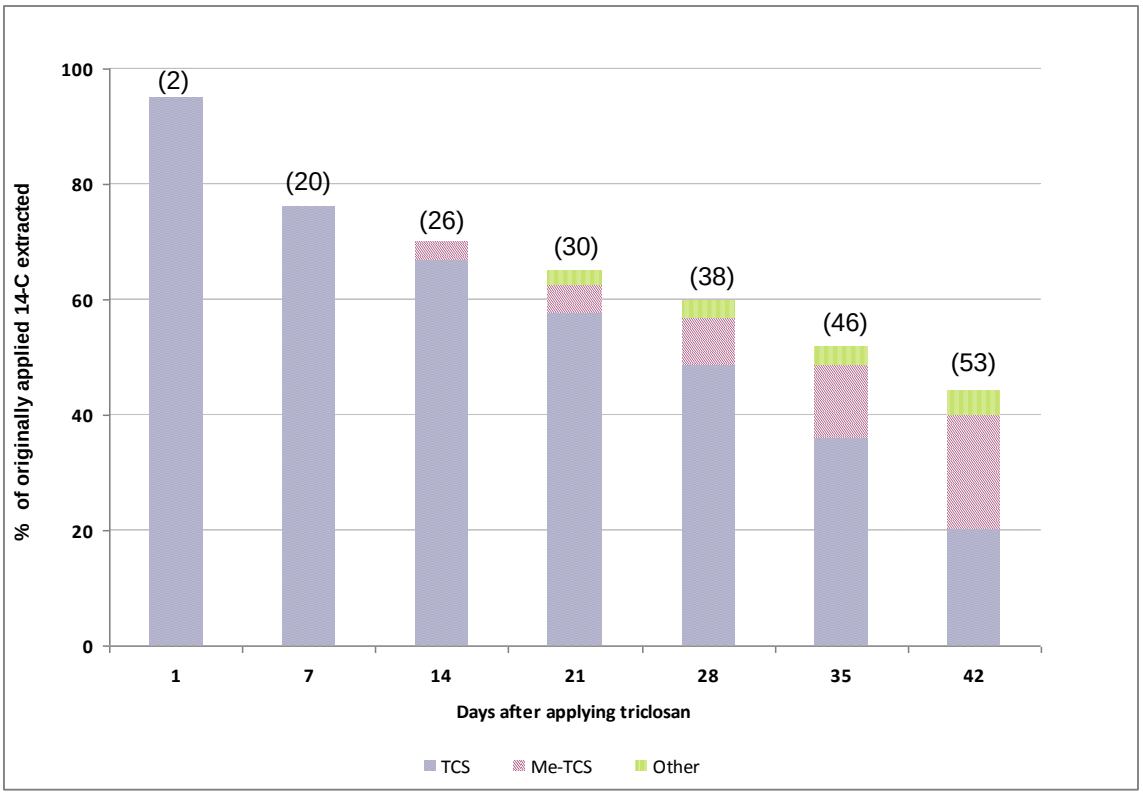



**Figure 2-5 The amount of the originally applied $^{14}$C triclosan (TCS) extracted and the quantity present in parent form, as methyl-triclosan (Me-TCS) or in an unidentified form. The numbers in parenthesis represent the non-extractable fraction.**

## 2.5 Discussion

Organic chemicals in soil can be affected by a combination of biodegradation, transformation, volatilisation, photolysis, hydrolysis and the formation of bound

residues (Scheringer *et al*, 2006; Boethling et al., 2009). Triclosan volatilisation is likely to be negligible given its low Henry's law constant of 2.27 x $10^{-3}$ Pa $m^3$ $mol^{-1}$ (Lindström *et al.*, 2002). Some photolytic degradation is possible (Aranami *et al*., 2007), although this was minimised in this study by incubating the biometer flasks in the dark. Thus, the main pathways for triclosan dissipation in the experiment described here are likely to be transformation, mineralisation and the formation of non-extractable residues.

### 2.5.1 Triclosan Mineralisation

The results of this study suggest that complete mineralisation of triclosan was very slow in all soils. The temporal pattern of mineralisation could not be described using simple first order kinetics. This means that it is not possible to calculate a half life. Most of the mineralisation data could be better described using a two pool exponential model (double first order) with two pools with different half lives. The half lives for the labile pool were generally in the range 9-25 days for direct spiking treatments and about 40 days for the treatments where triclosan was introduced with sludge. The recalcitrant pool had much longer best-fit half lives of between 6-8 years for the direct spiking treatment and over 244 years in the sludge-associated treatments. Previously reported mineralisation half lives vary from between 577 days (Ciba, 2004) and 17-35 days (Springborn Laboratories, 1994).

Many studies have reported that triclosan is mineralised following first order kinetics (Al-Rajab et al., 2009; Cha and Cupples, 2010; Chen *et al*., 2011). However, the results of this study suggest that simple first order kinetics were inappropriate and that mineralisation could be better described by biphasic kinetics. In the first phase triclosan is mineralised fairly rapidly and in the second phase, much more slowly. The reduction in mineralisation rate could be due to the formation of more persistent metabolites such as methyl-triclosan which may explain the slower recalcitrant phase. Alternatively, it could be due to the formation of a bound residue in which some triclosan may exist untransformed but in a non-extractable state. The extent to which bound residues are irreversible is a matter of some debate (see Boethling et al., 2009, for example). Langdon *et al* (2011) also successfully applied a biphasic model to describe triclosan mineralisation. They observed $R^2$ values of 0.17 for a 1$^{st}$ order model compared to 0.58 using a biphasic model. They observed an initial $DT_{50}$ of between 1.2 and 6.3 days, with 51% of the originally applied triclosan remaining in the soil at the end of their 224 day study. Our work confirms that initial mineralisation is fairly rapid and the majority of the triclosan in the soil is relatively recalcitrant. There are several other examples of where biphasic models have been used to describe the dissipation of different organic compounds in soil (e.g. Hill and Schaalje, 1985; Ma *et al*., 2004; Sarmah and Rohan, 2011). The availability-adjusted first order model is a variation of the first order model, where it is assumed that dissipation follows first order kinetics but the availability of the compound decreases exponentially with time and the unavailable fraction is assumed to be totally recalcitrant (Wang *et al.,* 2006; Krogh *et al*., 2009).

Our observation that triclosan dissipates at different rates depending on how it is applied to the soil is also supported by other studies (e.g. Al-Rajab *et al,* 2009). It has been shown that triclosan dissipates rapidly when liquid sludge is applied compared with directly applying triclosan to soil or application with dried sludge pellets. This could be due to a higher nutrient content in the liquid which can catalyse degradation by stimulating triclosan-transforming microorganisms (Meade *et al*., 2001). Dry pellets, on the other hand, have less physical contact with the soil and a greater degree of sorption to the sludge than the soil, which will reduce triclosan diffusion from the pellets into the soil and, hence, reduce biodegradation. Another potential reason for differences in reported mineralisation rates between studies could be different incubation times. The study conducted by Al-Rajab *et al* (2009) only ran for 42 days whereas the study by Ciba (2004) was left until 50% of the starting material had mineralised (577 days). Our study was run for 180 days and showed that although mineralisation begins fairly rapidly, after approximately 40 days mineralisation was relatively slow. The OECD method 304A guidelines suggest that an experiment should not be run for longer than 64 days regardless of the amount of mineralisation observed as the soil is no longer representative of field soil samples.

### 2.5.2 Triclosan biodegradation

The behaviour of triclosan observed here was similar to that reported for other organic pollutants in soil. The low rate of mineralisation does not necessarily imply persistence of the parent compound. In fact, it is clear that triclosan begins to be

biologically degraded very rapidly to form methyl-triclosan. Microbial degradation seems to be the most important dissipation mechanism as no significant mineralisation was observed in the in the sterile soils. This has also been observed in other studies on triclosan (e.g. Ying *et al.,* 2007). Only 50% of the $^{14}$C originally applied was extractable in its parent form from Day 28 suggesting that the primary half-life ($DT_{50}$) of triclosan is in the region of 28 days. By the end of the study (Figure 2.5), only 20% of the applied $^{14}$C was extractable in parent triclosan form suggesting a $DT_{80}$ of about 42 days. This primary degradation rate is comparable to others in the literature. For example, Ying *et al.,* (2007); Reiss *et al., (*2009); Wu *et al., (*2007) report half-lives of 18, 35 and 58 days respectively. In our study, initial mineralisation was most rapid in the loamy sand soil and slowest in the heavy clay soil. There are several potential reasons for this. Triclosan is a hydrophobic compound with a Log $K_{oc}$ value of 4.8 (Reiss, 2002). It will, therefore, have a high affinity for the soil solid phase and the soil with the highest organic matter content (i.e. the clay soil) would be expected to sorb triclosan most strongly (Patra *et al*., 2008). Sorption will tend to reduce triclosan bioavailability (aqueous phase concentration) which will generally reduce microbially mediated degradation.. In addition, the heavier soils will have a higher fraction of small pores, which may offer some spatial protection for triclosan from microbial attack (e.g. Balesdenta *et al*., 2000; Sleutel *et al*., 2012).

### 2.5.3 Non-extractable residues

Bound or non-extractable residues are terms used to describe chemicals which cannot be extracted by methods which do not significantly change the chemical

nature of these residues (EEC directive 91/414/EEC; Boethling *et al*., 2009), or that which remains in the soil after exhaustive sequential extractions (Gevao *et al.,* 2005). Triclosan appears to form bound residues fairly rapidly in soils (implied by the difference between the $^{14}$C in combusted samples and the extractable fraction.

Triclosan was seen to form bound residues as quickly as Day 7 with 20% of the $^{14}$C being non-extractable (Figure 2.5). The longer compounds reside in soil, the tighter they tend to be held due to several processes including the formation strong covalent bonds (Haider *et al,* 1992) or physical entrapment of the compound in the soil organic matter or mineral matrix (Pignatello and Xing, 1996; Fu *et al.,* 1994). By Day 42, more than 50% of the $^{14}$C was non-extractable, although the nature of the non-extractable residue was not identified. Bound residues could represent a significant fate for triclosan in soils amended with biosolids. Recent studies have shown that between 51 and 60 % of applied triclosan can become recalcitrant in the laboratory (Langdon *et al*., 2011) and field (Al-Rajab *et al.,* 2009) respectively. These reported bound residue values confirm the findings of our study, where approximately 60% of the $^{14}$C applied was non-extractable by the end of the study. In the study of Al-Rajab *et al. (*2009) bound residues appeared to form quicker in soils receiving liquid biosolids compared with dry biosolids or triclosan in water, although the bound residue fraction after 30 days was similar in the triclosan in water treatment. In the soil receiving dry biosolids bound residue formation was much slower less extensive compared to the other treatments. In our study, there was no

apparent difference between the bound residue formed in soils directly receiving triclosan and in those receiving triclosan via dry biosolids.

It can be argued that bound residues are not bioaccessable or bioavailable to soil microbes, thereby eliminating the original issue of toxicity. Bound residues also prevent leaching and losses in overland flow, except when particles are mobilised (Semple, 2009). However, bound residues can be temporary and remobilisation is possible if conditions in the soil change, such as moisture content, temperature and pH (Barriuso, *et al.*, 2008). In some cases it is possible that some of the bound residue represents $^{14}$C from completely degraded triclosan which has been assimilated into the microbial biomass. Federle *et al.* (2002) reported that up to 6% of applied triclosan could be incorporated into the microbial biomass.

**Conclusions**

In this study was triclosan was shown to mineralise relatively slowly in a laboratory incubation. Less than 8% mineralisation was observed over a 6 month period. This was slightly lower than the rate expected based on previously reported degradation half-lives, although the rate of primary biodegradation was shown to be much faster. Triclosan mineralisation could be reasonably well explained by a two-pool biphasic model. The first phase explained an initially rapid mineralisation and the second phase described the slower mineralisation rate observed later, which was probably due to a combination of the formation of more persistent metabolites (i.e. methyl-

triclosan) and bound residue. Triclosan was shown to degrade to methyl-triclosan within 14 days of triclosan application. After 28 days, only 50% of the $^{14}$C was extractable in its parent form indicating a primary $DT_{50}$ of 28 days. This is very similar to the mineralisation half-lives in the rapidly degrading pool. The formation of bound-residues is a significant fate process for triclosan and represented in excess of 60% of the applied $^{14}$C at the end of the experiment. A more powerful and robust method of extraction and detection is needed in future to identify the form of these non extractable residues and the long term fate of triclosan applied to soils via the addition of sewage sludge. In order to fully validate the triclosan mineralisation reported here, a positive control should also have been established using a readily degradable substance, such as glucose.

# Chapter 3

## Analytical method development

## 3.1 Abstract

Triclosan is an antimicrobial agent used in a wide range of personal care products such as hand soaps and toothpastes as well as in materials such as chopping boards and toys. Due to its hydrophobic nature, triclosan is sequestered to sewage sludge during wastewater treatment, and can be applied to land via the sludge to soil route. However, relatively little work has been conducted on the fate and transport in soil of triclosan and its principle metabolites. In this paper a robust and reliable analytical method was developed to extract, detect and quantify both triclosan and methyl triclosan in soils and sewage sludge. Methyl-triclosan is a major metabolite of triclosan, which is more hydrophobic and persistent than triclosan itself in sewage sludge and soil. This was achieved using Accelerated Solvent Extraction (ASE), followed by Solid Phase Extraction (SPE) and Gas Chromatography Mass Spectroscopy (GC-MS). $^{13}C$ labelled internal standards were used to accurately quantify triclosan and methyl-triclosan recovery and detection. This method gave an extraction efficiency of approximately 94% for both triclosan and methyl triclosan from soil, and a detection limit of 1.65 ng $g^{-1}$. The method proved highly repeatable.

## 3.2 Introduction

Triclosan (5-Chloro-2-[2,4-dichloro-phenoxy]-phenol) is a broad spectrum antimicrobial agent and preservative, which is commonly found in home and personal-care products as well as being incorporated into polymers and fibres to make clothes, shoes, food chopping boards and sports wear (Aranami and Readman, 2007; Clayborn *et al*., 2011; Møretrø *et al*., 2011). It has been estimated that over 350 tonnes of triclosan are produced annually for European use alone (Singer *et al*, 2002). Studies that have been undertaken on various pharmaceuticals and personal care products indicate that levels are often higher in soil than in aquatic samples and there have been suggestions that there is greater potential for accumulation in soils in part due to longer residence times (Barron *et al.,* 2008). Triclosan has a low vapour pressure (Henry's law constant 2.27 $X10^{-3}$ Pa $m^3$ $mol^{-1}$), making it non-volatile and has a high octanol-water partition coefficient (Log $K_{ow}$), which indicates a high likelihood for partitioning to sewage sludge during the waste-water treatment process. Triclosan has been consistently found in digested sludge at concentrations ranging from 10 - 24.6 mg $kg^{-1}$ (Barron *et al*., 2008).

Methyl-Triclosan (5-chloro-2-(2, 4-dichlorophenoxy) anisole) is a known metabolite of triclosan. Whilst triclosan is an antibacterial agent, methyl-triclosan does not possess antibacterial activity and there are no known industrial applications. Compared to triclosan, methyl-triclosan is more lipophilic and environmentally persistent (Chu and Metcalfe, 2007; Coogan *et al*., 2007) and there have been suggestions that it has a high bioaccumulation potential (Ying and Kookana, 2007). Methyl-triclosan is commonly detected in surface waters and sewage effluent (Halden and Paull, 2005;

Lindström *et al*., 2002; McAvoy *et al*., 2002) and has recently been reported in sewage sludge and soils at high concentrations (Waria *et al.,* 2011). It is known to be formed by bacterial methylation of parent triclosan in the waste-water treatment plant (Chen *et al*., 2011; Balmer *et al*., 2004).

Many studies have looked at triclosan in the aquatic environments (Lindström *et al*., 2002; McAvoy *et al*., 2002; Sun *et al.,* 2011; Yu *et al*., 2011; Ramaswamy *et al*., 2011), but few of these report the simultaneous detection of triclosan and methyl-triclosan (Kantiani *et al.,* 2008; Gonzalez-Marino *et al*., 2011; Casas Ferreira *et al.,* 2011). The detection methods used in aqueous samples cannot be applied to solid samples such as soil and sewage sludge due to the complexity of the matrix (Heidler & Halden, 2007). Additional extraction steps are needed prior to analysis such as soxhlet (Bester, 2003; Ozaki *et al.*, 2011) and pressurised liquid extraction (PLE) also known as accelerated solvent extraction (ASE: Aguera *et* al., 2003; Burkhardt *et al*., 2005; Chu and Metcalfe, 2007; Cha and Cupples, 2009), Microwave-assisted extraction (MAE: Sanchez-Prado *et al*, 2010; Morales *et al*., 2005), centrifugation (Heidler & Halden, 2007), filtration (Halden and Paull, 2005) and sonication (Gatidou *et al.,* 2007; Geens *et al.,* 2009; McClellen and Halden, 2010) have also been employed.

In this paper we report a reliable, robust and sensitive method of simultaneously extracting triclosan and methyl-triclosan from solid matrices, such as soil and biosolids, followed by accurate quantification of both analytes. Various commonly

cited extraction and clean-up techniques were tested to determine the most sensitive and repeatable method with the ability to detect trace quantities of both compounds in soils and biosolids.

## 3.3 Method development

The method employed is illustrated schematically in figure 3-2

### 3.3.1 Soil samples

Soil was collected from an agricultural field on Silsoe farm, Bedfordshire, England. The soil had a sandy loam texture (63.72 % sand, 18.85 % silt and 17.43 % clay) with a pH value of 6.9 and a total carbon content of 2.34 % by mass, a total nitrogen content of 0.209 % by mass and a cation exchange capacity of 82.7 cmol + /kg. Soils had followed an arable rotation of peas and wheat for the past ten years and were known to have not received biosolids prior to sampling. Soils were air dried and sieved to 2 mm and refrigerated until use.

### 3.3.2 Sample extraction

Three different methods of extraction were compared to find the method of extraction with the highest overall recoveries. These were solvent extraction with and without sonication, followed by filtration and accelerated solvent extraction (ASE). The first two methods were relatively simple to apply. The ASE has previously been reported

as a very efficient extraction procedure for a range of organic compounds in soil and sediments (Camino-Sanchez *et al*., 2011; Vergnoux *et al*., 2011; Zhang *et al*., 2011).

### 3.3.2.1 Filtration and sonication

Fifty grams of the soil was placed into fifty 100 mL silanized glass bottles. To each soil, 1 mL of triclosan dissolved in methanol was added make a concentration of 1 mg $kg^{-1}$ dry soil and manually incorporated with a spatula. The bottles were left open in a fume cupboard for 2 hours to allow the methanol to evaporate. After this time the bottles were lidded and the soils kept at room temperature for 24 hours. Five different solvents (methanol, dichloromethane, acetonitrile, ethyl acetate and hexane) were compared. For each solvent, 20 mL was added to the soil aliquots in replicates of ten. Five replicates of each soil-solvent mix were placed in a sonic water bath for 30 minutes at a frequency of 40 kHz and ambient temperature, whilst the remaining five were left to rest. All fifty bottles were then placed on a side to side shaker for 30 minutes at 300 rpm and centrifuged at a relative centrifugal force (RCF) of 700 g for a further 15 minutes. The supernatants were then decanted into clean vials. Additionally, the 25 samples which were not sonicated were filtered through Millipore 0.45 µm membrane filters. The filter was washed with the appropriate solvent to optimise extraction. The samples were then kept refrigerated until required for analysis and further solid phase extraction.

### 3.3.2.2 Accelerated solvent extraction

The same five solvents were used with the ASE extractions. Five grams of the same sandy loam soil as described previously was spiked with triclosan in methanol to the

same nominal concentration as in the other extraction methods. This was then mixed with 20 g Ottawa sand (Fisher Scientific) to allow good solvent flow through the cells. The soil/sand mixture was then packed into 33 mL ASE cells (Dionex) and topped up with extra sand. A Dionex ASE 200 (Dionex, California, USA) was used for all extractions. A temperature of 100 ºC with a pressure of 1500 PSI (103.42 bar) and a flush time of five minutes was chosen with two static cycles of five minutes each. This method was adapted from Cha and Cupples (2009), the only difference was the addition of the second static cycle. The extractants were collected in glass vials and refrigerated until required.

### 3.3.3 Solid phase extraction

There are many different solid phase extraction (SPE) methods available in the literature, each using different cartridges and different solvents. The purpose of this study to extract triclosan with various cartridge and solvent combinations to minimise matrix effects, whilst optimising detection limits. In several studies it has been shown that triclosan is easily extractable using cation exchange cartridges (Chu and Metcalfe, 2007). However, methyl-triclosan requires anion exchange extraction (Boyd *et al*., 2004; Guo *et al.,* 2009). It was therefore decided to evaluate using Hydrophilic Lipophilic Balanced (HLB) cartridges (Oasis, Waters, 6 $cm^3$, 1 g) and C18 (Discovery Supelco, 6 $cm^3$, 1 g) cartridges only. Different extraction solvents, reported in the literature, were used with both cartridges to see which performed the best.

Four different methods cited in the literature were tested with both the C18 and HLB cartridges. All methods were tested first by extracting 100 µg $L^{-1}$ triclosan and 100 µg $L^{-1}$ methyl-triclosan from water acidified to pH 4 with glacial acetic acid to test the efficiency of the extraction in water and with no matrix effects. The acidification keeps triclosan in its stable neutral form and prevents sorption to the glassware. The same method was then repeated to test the suitability of the method to extract 100 µg $kg^{-1}$ triclosan and 100 µg $kg^{-1}$methyl-triclosan (dry weight) from soil. In total, 5 samples in water and 5 samples extracted from soil were eluted through each cartridge following each of the four methods (Fig 3-1). Samples were run in sequence, i.e. all of method one samples were extracted together through both cartridge types, followed by method two samples and so on.

*Method 1* (Tomlinson, 2007: MChem thesis)

Cartridges (both HLB and C18) were conditioned with 5 mL methanol, 5 mL (10%) methanol and 10 mL deionised water. Cartridges were attached to a vacuum manifold and water samples or extracted soil samples were run through the cartridges at a rate of 2 mL $min^{-1}$, allowed to dry for 1 minute and washed using 2 mL methanol/ water (25% v/v) to remove any polar co-extractives. The cartridges are then dried under full vacuum for 60 minutes. Elution was carried out using 2 x 2.5 mL methanol followed by 2 x 2.5 mL dichloromethane. The eluents were then combined, blown to dryness under a gentle nitrogen stream at 25˚C and reconstituted in 1 mL of hexane.

*Method 2* (Chu and Metcalfe, 2007)

Cartridges (both HLB and C18) were conditioned with 3 mL of methanol, 3 mL acetone, 3 mL DCM and 3 mL of hexane on a vacuum manifold, at a flow rate of 2 mL/min. The sample was loaded onto the cartridge, pulled through with 2 mL hexane, and 2 × 2 mL DCM t a rate of 2 mL $min^{-1}$, and allowed to dry for two minutes. The cartridge was then washed with 2 × 3 mL water and was then dried for a further 10 minutes. Elution was carried out using 3 x 3 mL of 50:50 methanol/acetone (v/v). Eluents were then blown to dryness under a gentle nitrogen stream at 25˚C and reconstituted in 1 mL of hexane.

*Method 3* (Ying and Kookana, 2007)

Cartridges (both HLB and C18) were first conditioned with 5 mL methanol followed by 5 mL deionised water, the sample then was loaded onto the cartridge and washed with 50 mL of 10% methanol in water (v/v). Triclosan was eluted using 2 x 4 mL ethyl acetate. Eluents were blown to dryness under a gentle nitrogen stream at 25˚C and reconstituted in 1 mL of hexane.

*Method 4* (Topp *et al*., 2008)

Cartridges (both HLB and C18) were sequentially conditioned with 6 mL of each of the solvents; ethyl-acetate/acetone (50:50), methanol and deionised water. The sample was then loaded onto the cartridge and eluted by adding 2 mL ethyl-acetate/acetone (50:50) which was allowed to remain in the cartridge for ten

minutes. This was repeated twice more to give an elution volume of 6 mL. Eluents are then blown to dryness under a gentle nitrogen stream at 25˚C and reconstituted in 1 mL of hexane.

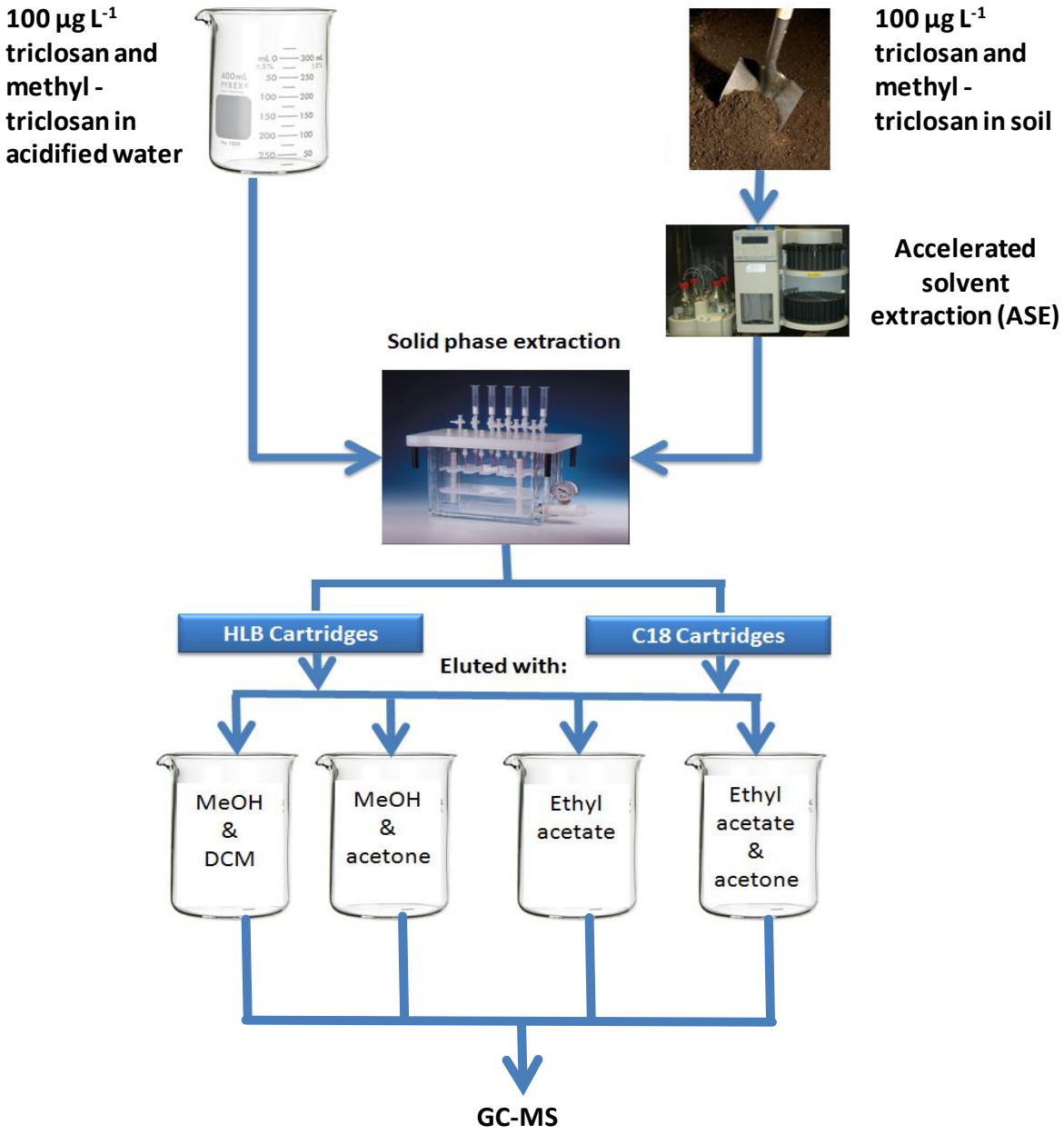



**Figure 3-1** Schematic of extraction method

### 3.3.4 Gas chromatography mass spectrometry

The GC used was an Agilent 6890 with Chemstation software. A Zebron ZB-5 HT column was used with dimensions, 30 m x 0.25 mm x 0.25 µm. The carrier Gas was helium set at a constant flow of 0.9 mL $min^{-1}$. Splitless injection was used to optimise triclosan delivery to the column at a temperature of 280ºC. To reduce triclosan sorption to the GC, a Siltek 5.2 mm standard deactivated glass liner. The injection volume was 1.0 µL, injected by auto-sampler. The oven temperature program was 120°C held for 1 minute, followed by a temperature ramp of 10°C $min^{-1}$ to 310°C which was then held for 5 minutes. The MS was programmed to have a solvent delay of 4 minutes, with an interface temperature of 310°C. The ionisation mode used was $EI^+$ electron impact and the method was run in full scan mode as well as Single Ion Monitoring (SIM). The triclosan ions had mass to charge ratio (*m/z*) of 218 and 288 with 290 *m/z* being detected as a confirmation ion. For the $^{13}C$ triclosan, 300 and 302 *m/z* were the ions detected and 230*m/z* the confirmation ion. The ions used to identify methyl-triclosan were 302 and 304 *m/z* and 252 *m/z* as the confirmation ion.

The GC-MS was tested using eight calibration standards in hexane with known triclosan concentration and $^{13}C$ labelled triclosan as an internal standard. The triclosan concentrations were 0.1, 1, 10, 20, 40, 60, 80 and 100 µg $L^{-1}$, each spiked with 100 µL of 500 µg $L^{-1}$ $^{13}C$-triclosan to give an internal standard concentration of 50 µg $L^{-1}$. The calibration graph (Figure 3-2) had an $R^2$ value of 0.99, for both triclosan and methyl triclosan, suggesting excellent instrumental accuracy.

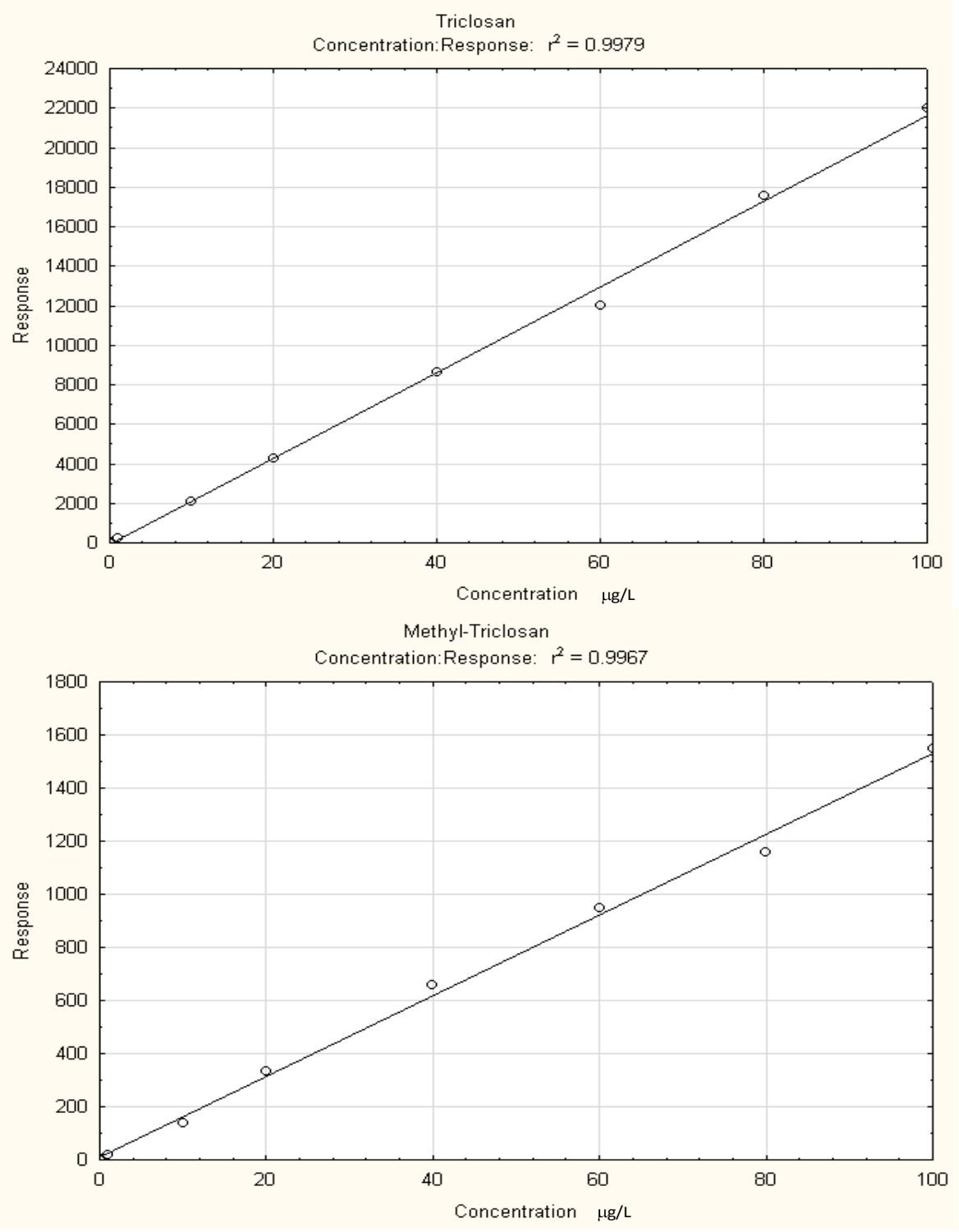



**Figure 3-2:** Calibration graphs for triclosan (TCS) and methyl triclosan (Me-TCS), both obtained by measuring the response against that of the $^{13}C$ triclosan internal standard.

### 3.3.5 Analysis of biosolid and sewage sludge cake

Biosolids (92.8% dry matter) and sewage sludge cake (24% dry matter) were sourced from a leading UK water company and analysed using the most effective extraction method to determine inherent triclosan and methyl-triclosan concentrations in each medium. Biosolid and sludge were dried for 24 hours at 105°C to remove all water and 5 replicates of each medium were prepared. Aliquots of 1g were spiked with 100 µL of 500 µg $L^{-1}$ $^{13}C$ triclosan and 100 µL of 500 µg $L^{-1}$ of $^{13}C$- methyl-triclosan both in nonane. Since concentrations of native (un-labelled) triclosan and methyl-triclosan in both media will vary, in order to estimate extraction efficiencies, $^{13}C$ methyl-triclosan would be the target analyte and $^{13}C$ triclosan was used as an internal standard.

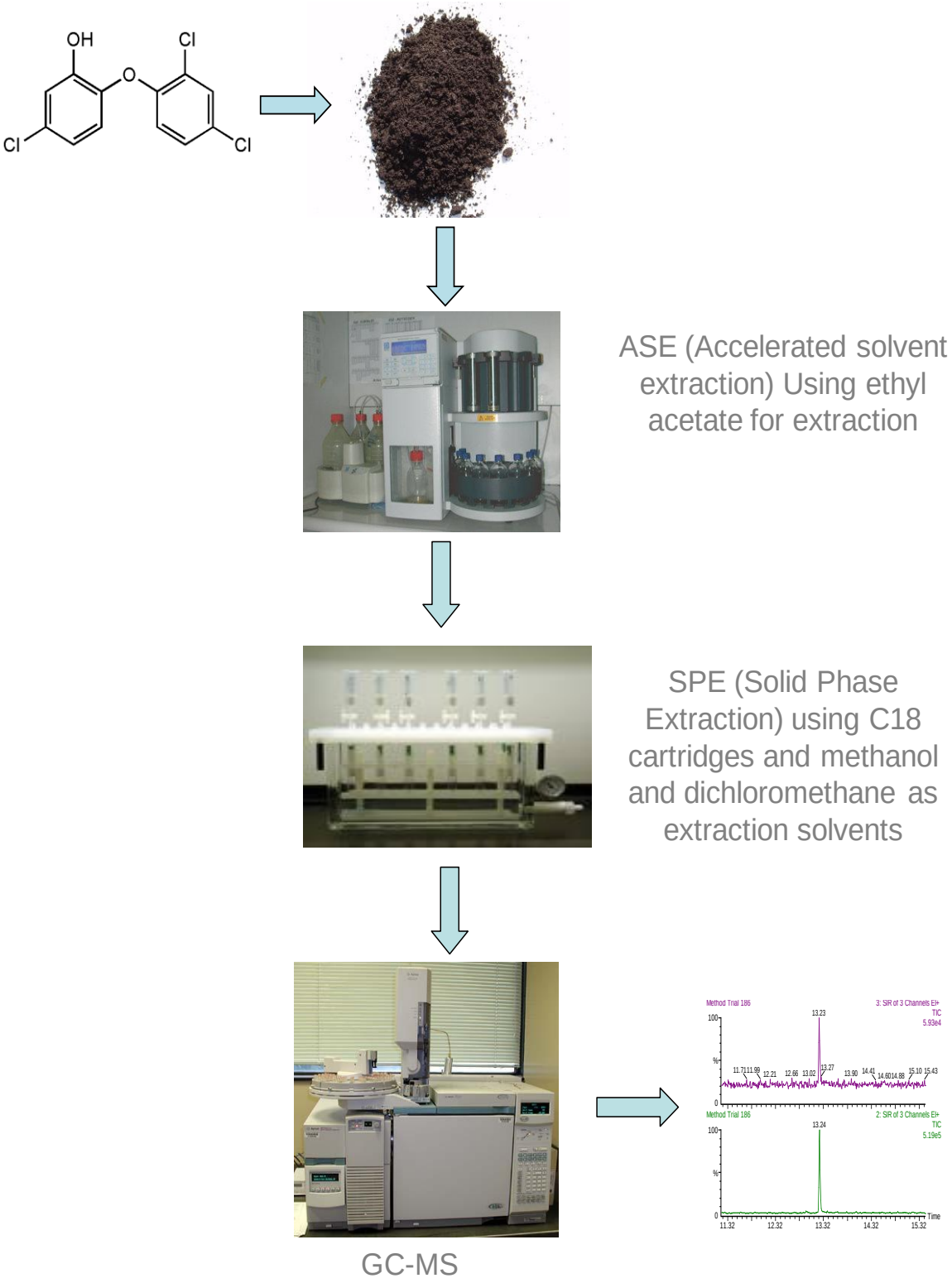



**Figure 3-3:** Summary schematic of the method used to detect triclosan and methyl-triclosan in soil.

## 3.4 Results and discussion

### 3.4.1 Sample extraction

All methods of extraction yielded very good extraction efficiencies for spiked triclosan in water and soil samples (Table 3-1). Both cartridges yielded good triclosan recovery from water ranging between 93.7 and 98.9% across the different methods. The best results were observed using the C18 cartridges eluted with methanol and dichloromethane. The HLB columns performed well with triclosan extraction but were less effective at retaining methyl-triclosan with recoveries ranging from 89-96.5%. This range compared to 97-98% recoveries observed with the C18 cartridges. The C18 cartridges yielded higher recoveries with all solvents compared to the HLB cartridges. The best solvents for methyl-triclosan extraction were ethyl acetate and acetone. However, this method also had the highest standard error.

Total extraction recoveries from soil, were slightly lower than with water. The highest extraction recovery was 95.3% which was obtained using the C18 cartridges. As in water, methyl-triclosan extraction was higher using C18 cartridges. The ethyl acetate/acetone and methanol/dichloromethane solvent methods had comparable extraction efficiencies; however as in water the ethyl acetate/acetone extractions saw higher standard deviations and standard errors. The method of using C18 cartridges and eluting with methanol/dichloromethane was chosen as the best and most reproducible sample clean up step with extraction efficiencies of 94.6 and 93.9% of triclosan and methyl-triclosan respectively removed from soil.

**Table 3-1:** Mean spiked triclosan and methyl triclosan recoveries plus the standard deviation (Stdev) and the standard error of the mean (SEM) for the two solid phase extraction cartridges (HLB and C18) for aqueous and soil samples

| | ***Triclosan*** | | | | | | ***Methyl-triclosan*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***HLB*** | | | ***C18*** | | | ***HLB*** | | | ***C18*** | | |
| ***Water samples*** | Mean | StDev | SEM | Mean | StDev | SEM | Mean | StDev | SEM | Mean | StDev | SEM |
| MeOH/DCM | 93.7 | 8.3 | 3.7 | 98.9 | 5.3 | 2.4 | 89.3 | 5.6 | 2.5 | 97.6 | 6.2 | 2.8 |
| MeOH/Acetone | 97.7 | 6.3 | 2.8 | 95.3 | 6.3 | 2.8 | 90.2 | 8.2 | 3.7 | 96.8 | 6.1 | 2.7 |
| Ethyl acetate | 97.9 | 5.9 | 2.6 | 95.3 | 7.5 | 3.4 | 90.1 | 8.8 | 3.9 | 97.2 | 8.25 | 3.7 |
| Ethyl acetate/Acetone | 96.9 | 6.6 | 3.0 | 98.6 | 6.8 | 3.0 | 96.5 | 8.5 | 3.8 | 98.1 | 11.25 | 5.0 |
| ***Soil Samples*** | | | | | | | | | | | | |
| MeOH/DCM | 92.3 | 8.9 | 4.0 | 94.6 | 7.25 | 3.2 | 92.3 | 8.91 | 4.0 | 93.9 | 5.3 | 2.4 |
| MeOH/Acetone | 94.6 | 10.66 | 4.8 | 91.3 | 8.66 | 3.9 | 90.2 | 9.25 | 4.1 | 90.3 | 6.02 | 2.7 |
| Ethyl acetate | 94.3 | 10.25 | 4.6 | 85.7 | 9.1 | 4.1 | 85.3 | 9.15 | 4.1 | 83.7 | 8.55 | 3.8 |
| Ethyl acetate/Acetone | 91.2 | 11.75 | 5.3 | 95.3 | 11.85 | 5.3 | 80.2 | 8.05 | 3.6 | 92.1 | 7.98 | 3.6 |

### 3.4.2 Sample clean-up

As expected, ASE was far superior in extracting triclosan and methyl-triclosan from soil compared with the other two methods (Table 3-2). Two solvents were most effective at extracting triclosan from soil: acetonitrile and ethyl-acetate. Both were suitable for the extraction of triclosan and methyl-triclosan using ASE. However, ethyl acetate resulted in slightly higher recoveries and lower standard deviations than extractions using acetonitrile. Ethyl acetate was therefore selected for all future extractions. Solvent addition with sonication and filtration did not yield high recoveries. The extractions also yielded variable results (Table 3-2). Coupled with the fact that these methods have high solvent volume usage and required more time

to complete extractions, means that both of these methods unsuitable for routine extractions.

**Table 3-2:** Mean triclosan and methyl triclosan extraction efficiency plus the standard deviation (stdev) and the standard error of the mean (SEM) for the five solvents (ethyl acetate, hexane, methanol, acetonitrile and dichloromethane) using three different extraction techniques, sonication, filtration and accelerated solvent extraction (ASE) followed by SPE with C18 cartridges and elution with methanol and dichloromethane (method one).

| | | *Triclosan* | | | *Methyl-triclosan* | | |
|---|---|---|---|---|---|---|---|
| | ***Solvent*** | ***Mean*** | ***stdev*** | ***SEM*** | ***Mean*** | ***stdev*** | ***SEM*** |
| ***Sonication plus Centrifugation*** | Ethyl-acetate | 66.94 | 9.1 | 4.1 | 42.15 | 15.3 | 6.8 |
| | Hexane | 64.73 | 7.0 | 3.1 | 43.11 | 11.3 | 5.0 |
| | Methanol | 64.18 | 1.9 | 0.9 | 46.99 | 14.8 | 6.6 |
| | Acetonitrile | 65.64 | 13.4 | 6.0 | 40.25 | 12.2 | 5.5 |
| | Dichloromethane | 62.28 | 15.8 | 7.1 | 39.55 | 18.7 | 8.3 |
| ***Centrifugation plus Filtration*** | Ethyl-acetate | 58.94 | 17.8 | 7.9 | 48.25 | 17.2 | 7.7 |
| | Hexane | 46.15 | 12.2 | 6.5 | 44.44 | 18.2 | 8.1 |
| | Methanol | 52.19 | 9.3 | 4.2 | 42.15 | 16.6 | 7.4 |
| | Acetonitrile | 58.18 | 9.6 | 4.3 | 47.36 | 12.6 | 5.6 |
| | Dichloromethane | 43.62 | 15.1 | 6.8 | 40.25 | 9.1 | 4.1 |
| ***ASE*** | Ethyl-acetate | 94.60 | 5.3 | 2.2 | 93.9 | 6.2 | 2.8 |
| | Hexane | 81.76 | 5.5 | 2.5 | 89.11 | 5.9 | 2.6 |
| | Methanol | 84.19 | 7.2 | 3.2 | 88.64 | 4.8 | 2.1 |
| | Acetonitrile | 92.28 | 5.8 | 2.6 | 96.87 | 4.9 | 2.2 |
| | Dichloromethane | 90.29 | 7.0 | 3.1 | 91.45 | 6.6 | 3.0 |

### 3.4.3 Method limits

The method detection limit (MDL), which combines the ASE, SPE and GC-MS steps, was determined by analysing seven samples with concentrations near the expected limit of detection. In this case this was 1 ng $g^{-1}$. The standard deviation of these values (0.175) was multiplied by the by the value of t for a 99% confidence interval. The value of t for 6 degrees of freedom (*n*-1) is 3.14. The MDL was therefore 0.55 ng $g^{-1}$. The limit of quantification (LOQ) is assumed to be 3 times the MDL (Corley, 2003). The LOQ was 1.64 ng $g^{-1}$. The relative standard deviation for MDL determination was 17%. When compared with other reported LOQs in the literature (Table 3-3), the method developed here, had excellent sensitivity and repeatability with a lower LOQ than other GC-MS methods reported elsewhere allowing for more reliable trace analysis. The increase in sensitivity could be due to better extraction efficiencies and cleaner extractants resulting in fewer matrix effects. LC-MS methods reported elsewhere (Lozano et al., 2010; Cha and Cupples, 2009), generally have lower limits of detection and quantification than those obtained using GC-MS. This is due to non-volatile nature of triclosan and due to the fact that it contains hydroxyl functional groups that can sorb to the GC injection inlets and the columns. However LC-MS is an unsuitable tool for the simultaneous determination of triclosan and methyl triclosan owing to the similar fragment ions formed during ionisation.

Derivatisation is a possible way to lower detection limits, however derivatising involves an extra chemical step, which can lead to problems such as an increased risk of human error, increased cost, increased analysis time and an increased potential for contamination. Recoveries of non-derivatised triclosan in the literature

have varied from 68-84% in river water (Aguera *et al*., 2003) to between 88-100% (Bester, 2005). However some studies where derivatisation has been employed have had a lower recoveries than that presented here, for example derivatisation with BSTFA (N,Ois(trimethylsilyl)trifluoroacetamide) saw recoveries of 60% (Boyd *et al.,* 2003). In view of the fact that acceptable LOD and LOQ were obtained without derivatising, combined with good recovery and repeatability, we propose that derivatisation is not required.

**Table 3-3:** A comparison of the LOQ obtained in this study compared to that reported by others for solid matrices.

| ***Matrix*** | ***Extraction*** | ***Analysis*** | ***LOQ (ng $g^{-1}$)*** | ***Authors*** |
|---|---|---|---|---|
| **Sewage sludge** | Soxhlet, SPE | GC-MS | 4 | Bester, 2003 |
| **Sewage sludge** | ASE, SPE | GC-MS | 5 | Ying and Kookana, 2007 |
| **biosolids** | ASE, SPE | GC-MS | 49.6 | Kinney *et al* ., 2006 |
| **biosolids** | ASE, SPE | GC-MS | 12.5 | Xia *et al* ., 2010 |
| **Soil** | ASE, SPE | LC-ESi-MS-MS | 2 | Lozano *et al* ., 2010 |
| **Soil** | ASE | LC-ESi-MS-MS | 0.05 | Cha and Cupples, 2009 |
| **Soil** | ASE, SPE | GC-MS | 1.65 | Present study |

**SPE – Solid phase extraction; ASE – Accelerated solvent extraction; GC-MS – gas chromatography mass spectrometry; LC-ESi-MS-MS – Liquid chromatography electrospray ionisation with tandem mass spectrometry; LOQ – Level of quantification.**

### 3.4.4 Analysis of sewage sludge and biosolids

Both triclosan and methyl-triclosan were observed in the sewage sludge and biosolid samples analysed here. Concentrations of triclosan in sludge pellets ranged from 15-35 mg $kg^{-1}$ with a mean concentration of 22 mg $kg^{-1}$, compared to 20 to 55 mg $kg^{-1}$

reported by Heidler and Halden (2007) with a mean concentration of 30 mg $kg^{-1}$ from sewage sludge sampled from Boston, USA. These concentrations are very similar to predicted concentrations from the Terrestrial model based on the technical guidance document (TGD, 2003), of 17-34 mg $kg^{-1}$ based on a per capita triclosan use of 2.75 mg $cap^{-1}$ $d^{-1}$ (Capdevielle *et al*., 2008). Concentrations measured in the sludge cake were slightly higher than those in pellets with concentrations ranging from 11 – 44 mg $kg^{-1}$ and a mean of 28 mg $kg^{-1}$. The variability suggests that the triclosan in the sludge cake is less homogenous than those in pellets and has the potential to have higher triclosan concentrations. Methyl-triclosan was also detected in both biosolids pellets and in sludge cake but at much lower concentrations than triclosan. Concentrations ranged between 18 to 25 µg $kg^{-1}$. The extraction efficiency of the $^{13}C$ methyl-triclosan was 90.15% (standard deviation of 9.25) in the sewage sludge and 82.36% in the pellets. These results are comparable with or higher than other recoveries observed in the literature (e.g. 78-106%, Morales *et al*., 2005; 75%, Chen *et al.,* 2011; and 97.7% Chu and Metcalfe, 2007) for sludge samples and (84%, Langdon *et al*., 2011; and 116%, Al-Rajab *et al*., 2009) for biosolids.

## 3.5 Conclusions

A reliable and repeatable method for the simultaneous detection of low level concentrations of triclosan and methyl-triclosan has been developed. The method detection limit was 0.55 ng $g^{-1}$ with a LOQ of 1.6 ng $g^{-1}$ in all three matrices. In the optimal method triclosan and methyl-triclosan were extracted from the soil or sludge with ethyl acetate using accelerated solvent extraction (ASE). After extraction the extractant was cleaned up using C18 SPE cartridges with elution with

dichloromethane and methanol. Separation and quantification were carried out using GC-MS sing an internal standard of $^{13}C$ labelled triclosan. The extraction efficiency of this method was approximately 94% for both triclosan and methyl triclosan in soil and around 90% in biosolids. Although LC-MS has a greater sensitivity for triclosan detection, it is currently unsuitable for the simultaneous detection of triclosan and methyl triclosan due to the formation of the same fragment ions after ionisation. This means that in order to increase sensitivity the GC-MS method needs to be optimised. This could be further increased by derivatising triclosan to reduce sorption to the GC equipment.

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# Chapter 4

## Fate of triclosan in field soils receiving sewage sludge

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## 4.1 Abstract

The widely-used anti-microbial substance triclosan can partition to sewage sludge during waste water treatment and subsequently transfer to soil when applied to land. Here, we describe the fate of triclosan in a one-year plot experiment on three different soils (loamy sand, sandy clay loam and clay) receiving 50 tonnes dry solids $ha^{-1}$ of dried sludge pellets. This application rate was higher than general application rates in Europe, but in line with rates applied in the USA. Triclosan and methyl triclosan concentrations were measured in soil samples collected monthly from three depths (0-10, 10-20 and 20-30 cm) in each plot. A large fraction of triclosan loss appeared to be explained by transformation to methyl triclosan. The most rapid transformation occurred during the summer (between June and September). Triclosan translocation to lower horizons was observed in all three soils but was most pronounced in the sandy soil and least pronounced in the clay soil, reflecting differences triclosan mobility caused by pH and soil organic matter content as well as differences in drainage and associated leaching. After 12 months approximately 8%, 12% and 10% of the initial triclosan was recovered in the top 30 cm of soil in the loamy sand, sandy clay loam and clay soils, respectively. However, between 39 and 66% of the triclosan applied was recovered as methyl triclosan. Most of the chemical recovered at the end of the experiment (both triclosan and methyl-triclosan) was still in the top 10 cm layer, although there was translocation to lower soil horizons in all three soils. Between 16.5 and 50.6% of the applied triclosan was unaccounted for at the end of the experiment either as a consequence of degradation or the formation of non-extractable residues.

**Key Words:** Soil, Triclosan, Methyl-triclosan, Degradation, Leaching

## 4.2 Introduction

Triclosan is a broad spectrum anti-microbial compound commonly used in personal-care products such as toothpaste and soaps. It has become a compound of increasing interest over recent years due to its frequent detection in aquatic environments at concentrations that sometimes exceed screening-level effect thresholds in river reaches with low dilution (e.g. Capdevielle *et al*., 2008; Price *et al*., 2010). Reported triclosan concentrations in treated waste water range between 0.04 $\mu gL^{-1}$ (McAvoy *et al.,* 2002) and 2.5 $\mu gL^{-1}$ (Yu *et al.,* 2006) depending on the treatment processes employed (Reiss *et al.,* 2002). Although waste-water treatment can remove up to 98% of triclosan from the effluent, a high fraction (typically 30-50%) will sorb to sewage sludge (biosolids). When sludge is applied to land, soils can be exposed to triclosan. The UK alone produces over one million tonnes of sludge (dry solids) annually, of which 62% is applied to agricultural land (Water UK, 2006). Analyses of biosolids for triclosan worldwide suggest that concentrations typically range between 0.5 and 55 mg $kg^{-1}$ (Bester, 2003; McAvoy *et al.,* 2002; Heidler and Halden, 2007; McClellan and Halden, 2010), although the USEPA Targeted National Sewage Sludge Survey (TNSSS) (USEPA, 2009) found triclosan at concentrations of up to 133 mg $kg^{-1}$ dry weight, with mean concentrations of 12 ± 18 mg $kg^{-1}$ in U.S sludge samples. It is notable that very few studies have reported triclosan concentrations in soil following sludge incorporation. Measured concentrations have been reported previously to range between 0.052 and 0.206 mg $kg^{-1}$ dry wt (Xia *et al*., 2010; Lozano *et al*., 2009), although these measurements were taken 7 and 12 months after application respectively. Modelled concentrations in soil have been estimated at 4.5 mg $kg^{-1}$ dry wt (Fuchsman *et al*., 2010).These concentrations compare with a soil microbial predicted no-effect concentration

(PNEC) of 1 mg $kg^{-1}$ dry wt (Waller and Kookana, 2008), a crop PNEC of approximately 1 mg $kg^{-1}$ dry wt and an earthworm PNEC of 1026 mg $kg^{-1}$ dry wt (Wuethrich, 1990), suggesting that some effects may be possible if soils receive very high doses. Indeed, Butler *et al* (2011) have shown that both microbial basal respiration and substrate induced respiration can be inhibited by triclosan addition, although at much higher nominal concentrations than those expected in field soils. It is also important to obtain a thorough understanding of the fate and translocation of triclosan in agricultural soils receiving sludge in order to address concerns about its potential persistence, bioaccumulation and toxicity in the terrestrial environment and to assess potential transport to ground and surface waters.

Currently little information is available on triclosan transport in soils. Factors that are likely to influence mobility include soil type (texture, pH, cation exchange capacity and organic matter content), hydrological and thermal regimes, the type of biosolid applied, the application rate, application timing and the application method. Topp *et al.* (2008), for example, reported that no triclosan could be detected in surface runoff when biosolids were injected into soil but when sludge was applied to the soil surface triclosan was detected in runoff up to 266 days after application. Triclosan has also been measured in soil drainage water via tile drain monitoring after sludge application (Edwards *et al*., 2009), suggesting that it has some potential for leaching. Although it is very hydrophobic, translocation is still possible via the transport of colloids through soil macropores, as well as via bioturbation and leaching of dissolved-phase chemical in pore water. Lapen *et al.* (2008) have shown that triclosan leaching can be reduced by adding dewatered biosolids as opposed to

liquid. In another study, Xia *et al.,* (2010) measured triclosan in soils that had received biosolids annually for 33 years. They reported that between 49-64% of the extractable triclosan was located at depth (30-120 cm) indicating substantial translocation. Notwithstanding the clear potential for triclosan transport in or over soil, the loss of triclosan in runoff and leachate is usually a very small fraction of the mass applied (<< 1%) suggesting that most of the chemical remains near the soil surface or is degraded (Sabourin *et al.,* 2009).

Several studies have reported dissipation of triclosan in field soils. For example, Lozano *et al.* (2010) observed concentrations between 23.6 and 66.6 $\mu g\ kg^{-1}$ dry wt in U.S soils one year after a single application of sludge. Concentrations decreased over time and were between 4.1 and 4.5 $\mu g\ kg^{-1}$ dry wt 16 months after application. Concentrations were slightly higher (*ca.* 10 $\mu g\ kg^{-1}$) in soil that had received repeated sludge applications. Cha and Cupples (2009) have also presented evidence for triclosan dissipation. Fields sampled between three months and four years after biosolids application contained lower triclosan concentrations than anticipated and when sampled a year later, concentrations had decreased further. A few studies have attempted to follow triclosan degradation explicitly and a range of degradation rates have been reported under different conditions. Ying *et al.* (2007), for example, reported a degradation half-life for triclosan of 18 days in a laboratory study in which triclosan was applied directly to a loam soil, although no degradation was observed in oxygen-deprived soils. Waria *et al*., (2011) reported degradation half-lives of 78 days for a silty clay loam and 421 days for a fine sand soil in a laboratory incubation study. Other reported half-lives include 13.1- 32.5 days (Xu *et*

*al.,* 2009) and 107 days (Lozano *et al.,* 2010) in the field and 13.5 days (Xu *et al*., 2008) and 2.5-35 days (Reiss, 2009) in the laboratory. Although different dissipation rates under different soil properties and environmental conditions should be expected, Cha and Cupples (2009) failed to correlate triclosan concentrations with soil characteristics. Mineralisation studies have shown that triclosan degradation can increase when applied with liquid biosolids compared to direct addition, particularly when compared with the addition of dewatered biosolids (Al-Rajab *et al.,* 2009).

Methyl-triclosan (Me-TCS) is a known metabolite of triclosan. Although it does not possess antibacterial properties, it is more lipophilic and potentially more environmentally persistent than the parent compound (Chu and Metcalfe, 2007; Coogan *et al*., 2007) and has a high bioaccumulation potential in aquatic organisms (Ying and Kookana, 2007). Methyl-triclosan is formed by way of O-methylation when a methyl group is attached to the hydroxyl group on the triclosan molecule, increasing lipophilicity (Häggblom *et al*., 1989; Allard *et al*., 1987). In general, the anisole products of O-methylation also have a tendency to have increased toxicity (Goswami *et al*., 2007). It is produced exclusively by aerobic biodegradation of the parent compound (Chen *et al.,* 2011) and has been reported in treated waste water effluent (Bester, 2003) and river water (Coogan *et al*., 2007). Thus far, the only measured concentrations of Me-TCS in biosolids or soils receiving biosolids have been those reported by Waria *et al* (2001), which reported a significant Me-TCS formation in laboratory-incubated soils.

In this study, the fate and translocation of triclosan and Me-TCS in agricultural soils receiving sewage sludge pellets under field conditions is reported. A primary objective was to better-understand degradation and translocation processes by simultaneously monitoring the fate of the parent compound and a principal biotransformation metabolite.

## 4.3 Methods

*Field selection*

The experiment was conducted at Silsoe farm in Bedfordshire, UK. Three different soils with different textures were selected (clay, loamy sand and sandy clay loam). All soils had not previously received sewage sludge and were in a similar arable rotation (dominated by winter wheat and peas for over 20 years prior to the field experiment). The soils at each site were analysed for texture, organic matter content, pH, cation exchange capacity and water holding capacity (Table 1).

*Plot preparation*

Within each field, three replicate plots (3 x 3 m) were established. A control plot was also established in each field which received no sewage sludge. Sewage sludge was provided by a major UK water company in the form of heat-treated biosolid pellets from a commercial operation. This common procedure produces pellets which are ultra heat treated to remove harmful pathogens but this process is unlikely to remove chemical contaminants. The pellets were applied in September 2009, at rate of 50 tonnes dry mass $ha^{-1}$ (5 kg $m^{-2}$) two days after the crop (Winter wheat:

Triticum aestivum) had been drilled. This rate is approximately six times higher than the average European application rate of 8 tonnes $ha^{-1}$ (Hughes et al., 2008). However, the rate is still realistic compared with high applications in the US, where typical sludge applications range from 5-50 tonnes $ha^{-1}$ on agricultural soils (Fuchsman et al., 2010). The sludge was manually applied and manually incorporated into the top layer of the soil to a depth of 10 cm.

*Sampling*

Four weeks after sludge application and, thereafter, every month, five soil cores were collected in each plot at three depths (0-10 cm, 10-20 cm and 20-30 cm) using a 3 cm diameter tube auger. Sampling points were selected at the apices of a virtual "W" over the plot with a change in "W" orientation on each sampling occasion to attempt to account for spatial variability. Soil samples collected in each plot at each depth were bulked to form a single representative sample for the plot. All samples were air-dried, 2 mm sieved and tested for pH (BS ISO 10390:2005), gravimetric moisture content and organic matter content (BS EN 13039: 2000). Previous recovery studies confirmed that there was no sample loss during the drying and sieving stage (data not shown). In addition, monthly mean air temperature ($T$) was derived from daily air temperature data (MIDAS Land Surface Stations, UK Met Office). Volumetric water content ($\theta_v$) was calculated from gravimetric moisture content ($\theta_m$) by multiplying the bulk density.

**Table 4-1 Basic properties the three soils used in the experiments (EC – electrical conductivity, CEC – Cation exchange capacity, TOM – Total organic matter, TC – total carbon, WHC- water holding capacity).**

| Soil property | Loamy Sand | Sandy clay loam | Clay |
|---|---|---|---|
| Soil series name | Bearsted | Evesham | Lawford |
| Grid reference | 26˚25'W, 52˚25'N | 25˚56'W, 52˚12'N | 26˚27'W, 52˚33'N |
| pH | 8.2 | 6.6 | 7.1 |
| EC µS/cm | 72.8 | 82.7 | 55.9 |
| CEC cmol+/kg | 6.6 | 13.5 | 13 |
| TOM % | 3.95 | 5.94 | 7.625 |
| Water content % | 0.98 | 1.31 | 10.22 |
| TOC | 1.595 | 2.392 | 2.694 |
| TC | 1.714 | 2.348 | 2.778 |
| Sand % | 71.9 | 35.96 | 19.4 |
| Silt % | 16.4 | 30.12 | 24.16 |
| Clay % | 11.7 | 35.56 | 56.05 |
| Bulk Density (kg/m$^3$) | 1250 | 1300 | 1190 |
| Total Nitrogen % | 0.159 | 0.209 | 0.285 |
| Max WHC (% m/m) | 43.41 | 58.02 | 74.52 |
| C/N ratio | 10.8 | 11.2 | 9.7 |

**Standard methods were employed (Soils were dried, sieved to < 2 mm and stored following BS 7755 Section 2.6: 1994 and BS ISO 11464 2006. Soil pH was analysed following BS ISO 10390:2005; Total organic matter was obtained via loss on ignition (BS EN 13039: 2000); Total carbon and total nitrogen content was calculated after elemental analysis (BS 7755 section 3.8 and BS EN 13654-2 2001, respectively). Particle size distribution, electrical conductivity and cation exchange capacity were all obtained using BS 7755 (sections 5.4:1998, 5.5: 1999, 3.4: 1995 and 3.12: 1996 respectively).**

*Analytical method*

Each bulked sample was extracted and analysed for triclosan and Me-TCS in triplicate. Accelerated solvent extraction (ASE) was used to initially remove the

relevant compounds from the soil. 5 g of air dried soil was weighed into a 33 mL ASE cell and mixed with enough Ottawa sand (Fisher Scientific) to fill the cell. The sand is essential to allow solvent to flow through the cell without blocking the injector. Extraction was performed using 100% ethyl acetate at a pressure of 103 bars, and a temperature of 100˚C. Extractants were then cleaned up using solid phase extraction (SPE) using C18 cartridges (6 $cm^3$, 1 g) under vacuum. Each cartridge was pre-conditioned with 5 mL methanol, 5 mL (10%) methanol and 10 mL water, the cartridges were then allowed to gently air dry by under vacuum for 10 minutes. The sample was then pulled through the cartridge at 10 mL $min^{-1}$ with the rate controlled by adjusting the vacuum pressure. Each cartridge was allowed to dry for 1 minute and washed with 2 mL methanol / water (25% v/v) to remove any polar co-extractives. The cartridge was then dried under full vacuum for 60 minutes. Elution was carried out using 2 x 2.5 mL aliquots of methanol followed by 2 x 2.5 mL aliquots dichloromethane. The eluents were combined and blown to dryness under a gentle nitrogen stream at 40˚C. Fifty µL of 1 mg $L^{-1}$ $^{13}C$ labelled triclosan in nonane was used as an internal standard and added with 950 µL of hexane to reconstitute each extract which was then transferred to a GC sampling vial ready for analysis.

An Agilent 6890 GC was used, equipped with Chemstation software and a Zebron ZB-5 HT, 30 m x 0.25 mm x 0.25 µm column. The carrier gas was helium set at a constant flow of 0.9 mL $min^{-1}$. Splitless injection was used to optimise triclosan delivery to the column, at a temperature of 280 ºC. To reduce triclosan sorption to the GC, a Siltek 5.2 mm standard deactivated glass liner was used. A volume of 1.0

µL was injected by auto-sampler. The Oven Temperature Program was as follows: 120 °C held for 1 minute, followed by a temperature ramp of 10 °C $min^{-1}$ to 310°C, which was then held for 5 minutes. The MS was programmed to have a solvent delay of 4 minutes, with an interface temperature of 310 °C. The ionisation mode used was $EI^+$ electron impact and the method was run in full scan mode as well as Single Ion Monitoring (SIM). The ions used for identification and conformation were 218, 288 and 290 for triclosan; 300, 302 and 230 for Me-TCS and 254, 302 and 304 for the $^{13}C$ labelled triclosan internal standard. Recoveries in the range 89-101 % of spiked triclosan at a concentration of 10 ng $g^{-1}$ dry soil were obtained, with a mean recovery of 94.3 %. The method limit of detection (LOD) was 1.4 ng $g^{-1}$ and the limit of quantification (LOQ) was 4 ng $g^{-1}$.

*Biosolid and control soil analysis*

Triclosan and Me-TCS concentrations were determined in biosolids prior to its application to the plots using the above method of extraction and quantification. Control soil samples were also taken on each plot and at each depth to determine background levels of triclosan and methyl-triclosan.

## 4.4 Results

### 4.4.1 Triclosan in the soil and sewage sludge

Triclosan and Me-TCS were not detected in any of the soil plots prior to sludge application or in control plot samples. The measured concentration ranges for triclosan and Me-TCS in the sludge pellets used ($n$ = 9) were 11.22-28.22 mg $kg^{-1}$ for

triclosan and 0.035-0.069 mg $kg^{-1}$ for Me-TCS (triclosan mean=21.9, SD=5.35mg $kg^{-1}$; Me-TCS mean=0.051, SD=0.012 mg $kg^{-1}$). The measured concentrations of triclosan and Me-TCS in the top 10 cm layer of each soil type over the course of the experiment are shown in Figure 4-1, along with key explanatory variables (pH, $\theta_v$ and $T$). The initial concentrations of triclosan ($C_0$) in this layer (Table 4-2) were in approximate agreement with the expected concentration ($C_{ini}$, mg $kg^{-1}$) derived from the mean measured concentration in sludge pellets, assuming zero background triclosan concentration in soil (e.g. Jackson and Eduljee, 1994):

$$C_{ini} = \frac{E.C_{biosol}}{\rho.z}$$

where $C_{biosol}$ is the mean biosolid concentration (mg $kg^{-1}$), $E$ is the application rate (kg $m^{-2}$), $\rho$ is the soil bulk density (kg $m^{-3}$) and $z$ is the mixing depth (m).

**Table 4-2 Predicted and measured concentrations of triclosan and methyl-triclosan applied to each plot (n=3, standard deviation for each plot in parenthesis).**

| | Predicted values (μg $kg^{-1}$) | | Measured values (μg $kg^{-1}$) | |
|---|---|---|---|---|
| Soil | TCS (SD) | Me-TCS (SD) | TCS (SD) | Me-TCS (SD) |
| Loamy sand | 880 (33.3) | 2.04 (0.52) | 914.9 (83.9) | 1.08 (0.08) |
| Sandy clay loam | 846 (32) | 1.96 (0.50) | 773.5 (77.5) | 2.19 (0.39) |
| Clay | 924 (35) | 2.14 (0.54) | 948.6 (39.7) | 2.17 (0.67) |

### 4.4.2 Triclosan and methyl triclosan in the top 10 cm of soil

In all three soils, triclosan concentrations in the top 10 cm layer decreased relatively slowly in the first nine months after sludge incorporation (October 2009). The rate of dissipation increased markedly in the summer months, after the June 2010 sampling. In the loamy sand soil 59% of the initial mass of triclosan was lost from the top 10 cm during the four month period between July and August. In contrast, the equivalent mass losses in the sandy clay loam and clay soils were 72 and 74% respectively over the same period. In parallel, there was a small but progressive increase in the concentration of Me-TCS to June 2010, followed by a more rapid increase in summer, suggesting that a significant fraction of the measured loss of triclosan was due to biotransformation of the parent compound to Me-TCS.

The period of most rapid triclosan transformation coincided with a period of low soil moisture content (approaching the permanent wilting point of each soil) and increased air temperature (see Figure 4-1). An apparent decrease in triclosan transformation was observed in October 2010. At this point all three soils were wetter and the temperature was lower (*T* was approximately 8°C), suggesting that the rate of biodegradation may have been limited by abiotic factors. In the clay soil, the period of rapid triclosan dissipation appeared to occur slightly later (in August 2010) than in the other two soils, possibly due to a delay in soil drying (see figure 1).

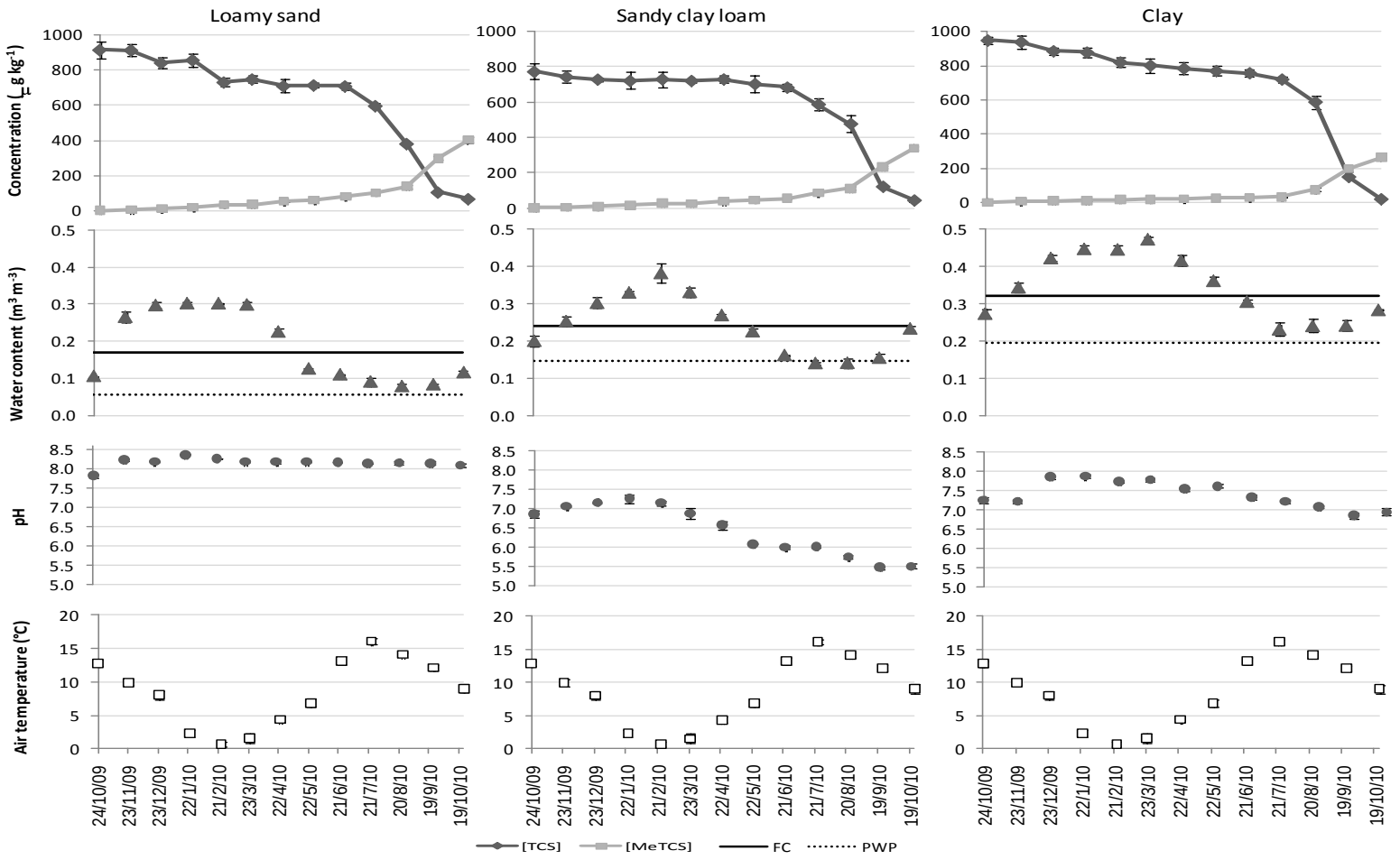


**Figure 4-1 The concentration of triclosan (TCS) and methyl-triclosan (Me-TCS) in the upper 10cm of each soil plotted along side the water content showing field capacity (FC) and permanent wilting point (PWP), pH and air temperature**

### 4.4.3 Triclosan Mass Balance

It is possible to estimate the mass of chemical remaining in the soil relative to that which was initially added; assuming that one mole of Me-TCS is derived from one mole of triclosan. This is shown in Figure 4-3. The mass remaining at the end of the experiment is shown in Table 4-3. The fraction of added chemical recovered as triclosan or Me-TCS after one year ranged from 49.4 % in the clay soil to 83.6 % in the loamy sand. Interestingly, the fraction of the final chemical mass recovered in triclosan form was similar for all three soils (18%, 12% and 10 % for the sand, loam and clay soils respectively), suggesting a high degree of primary degradation. However, a high fraction of the triclosan initially applied (66%, 64% and 39% in the case the sand, loam and clay soils, respectively) was recovered as Me-TCS at the end of the study period in the top 30 cm, suggesting that ultimate biodegradation rates were much lower. It is also noteworthy that approximately 50% of the applied chemical mass was extracted from the top layer of the loamy sand and sandy clay loam soils, in either parent or metabolite form (mainly as Me-TCS). Since Me-TCS is much less mobile in the soil than the parent compound, this material is unlikely to leach further except, perhaps, in colloidal form.

The fraction of chemical mass which could not be recovered at the end of the one year experiment may have been mineralised, or may have formed non-extractable (or bound) residues. The formation of bound residues is a common phenomenon in chemical fate studies in soils and sediments (Mordaunt *et al.,* 2004; Wanner *et al*., 2005; Boethling *et al*., 2009). They are operationally described as the chemical mass or radio-label remaining in the soil at the end of a fate study after exhaustive

sequential extractions (Gevao *et al.,* 2005). The mechanism of retention is often uncertain but may include covalent bonding (Haider *et al,* 1992), incorporation (polymerisation) of the chemical in soil organic matter (see Boethling *et al*., 2009) or physical entrapment of the compound in the soil organic or mineral matrix (Pignatello and Xing, 1996; Fu *et al.,* 1994) or the sludge pellets themselves (Hörsing *et al*., 2011). The use of radiolabelled compounds has also shown that a chemical can be incorporated into the soil biomass (Vyas *et al*., 1994; Federle *et al*., 2002). Bound residues are often assumed to be stable and biologically unavailable, at least in the short term and, thus, they will generally not be mobile (Semple, 2009), although it is possible that colloidal transport may translocate some bound material. It is also possible that some remobilisation could occur following substantial changes in soil moisture content, temperature or pH (Höllrigl-Rosta *et al.*, 2003), or through microbially mediated residue release (Roberts and Standen, 1991; Khan and Ivarson, 1990).

**Table 4-3** Mass balance calculations showing the amount of triclosan (TCS) and methyl-triclosan (Me-TCS) recovered in total in each soil layer in terms of mass and moles at the end of the experiment.

| | Loamy sand soil | | | Sandy clay loam soil | | | Clay soil | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mass (μg) | Moles (mMol) | % of applied | Mass (μg) | Moles (mMol) | % of applied | Mass (μg) | Moles (mMol) | % of applied |
| TCS applied | 914.9 | 3.164 | 100 | 773.5 | 2.679 | 100 | 948.6 | 3.284 | 100 |
| TCS 0-10cm | 69.11 | 0.239 | 7.6 | 44.33 | 0.153 | 5.7 | 20.22 | 0.07 | 2.1 |
| Me-TCS - 0-10cm | 403.8 | 1.33 | 42.0 | 339.92 | 1.12 | 41.8 | 264.84 | 0.872 | 26.6 |
| TCS 10-20cm | 36.39 | 0.126 | 4.0 | 19.78 | 0.068 | 2.5 | 49.69 | 0.172 | 5.2 |
| Me-TCS 10-20cm | 164.38 | 0.541 | 17.1 | 143.09 | 0.471 | 17.6 | 90.62 | 0.299 | 9.1 |
| TCS 20-30cm | 58.34 | 0.201 | 6.4 | 31.61 | 0.109 | 4.1 | 28.68 | 0.099 | 3.0 |
| Me-TCS 20-30cm | 62.55 | 0.206 | 6.5 | 40.29 | 0.133 | 5.0 | 32.45 | 0.11 | 3.3 |
| Total recovered (%) | | | 83.6 | | | 76.7 | | | 49.4 |
| Total TCS (%) | | | 17.9 | | | 12.3 | | | 10.4 |
| Total Me-TCS (%) | | | 65.6 | | | 64.4 | | | 39.0 |

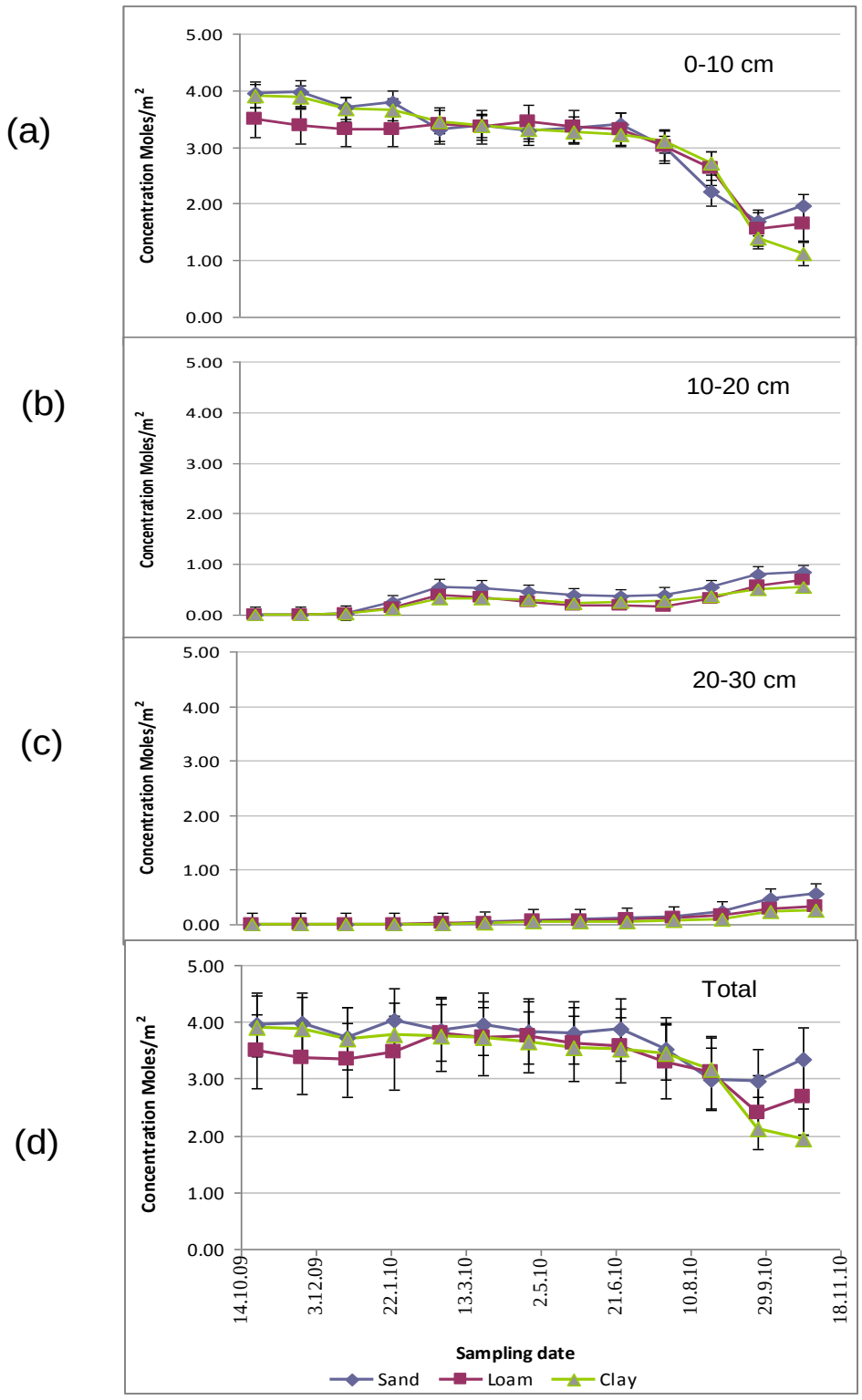


**Figure 4-2 The total amount (moles) of chemical recovered (triclosan and methyl-triclosan combined) for each soil in (a) 0-10 cm, (b) 10-20 cm, (c) 20-30 cm depth and (d) in all 3 layers.**

## 4.5 Discussion

Although Me-TCS is just one of several potential metabolites resulting from the biodegradation of triclosan, the data presented here suggest that it is the most significant one in the agricultural soils receiving sludge. Microbially mediated degradation of triclosan is the only known source of Me-TCS (Lindstöm *et al*., 2002). In our study, between 59 and 73% of triclosan dissipation occurred between July and October in all soil types (Figure 4-1). The close coincidence of declining triclosan concentration and increasing Me-TCS, particularly during the summer months provides convincing evidence for significant and seasonal biodegradation of triclosan. To some extent, this seasonality may account for some of the variation in triclosan half-lives reported in the literature (18 - 107 days: Ying *et al.,* 2007; Lozano *et al.,* 2010).

There are many factors that can affect the fate of organic chemicals in soil including abiotic factors (soil temperature, moisture content and land use), soil physical and chemical properties (e.g. texture, organic matter content, pH and porosity), chemical properties (molecular size and shape, degree of chlorination, pKa, aqueous solubility, hydrophobicity and vapour pressure) and the activities of soil biota and plants including adaptation of soil microorganisms to a particular chemical. Soil temperature can affect the rate of biodegradation by influencing soil microbial activity but will also affect partitioning. Neither triclosan nor Me-TCS are likely to be volatilised to any significant extent (the Henry's law constant for triclosan is $2.27 \times 10^{-3}$ Pa $m^3$ $mol^{-1}$), although the methyl group on the Me-TCS will increase its volatility. Therefore the main fate processes are likely to be biodegradation, leaching, sorption

to colloids – which can, themselves, be leached and sorption to the soil solid phase - which can be translocated via bioturbation (e.g. by earthworms).

Both triclosan degradation, as evidenced by the formation of Me-TCS, and translocation to lower depths appear to be seasonal – driven by a combination of changes in soil water content, temperature and drainage rate (itself, principally controlled by soil water content which, in turn, controls unsaturated hydraulic conductivity and hydraulic gradient). The rate of triclosan degradation was relatively low during the winter period. This is likely to have been limited by high soil water content (which was above field capacity for much of the winter in all three soils), as well as by low temperatures. Triclosan degradation is known to be strictly an aerobic process (Ying *et al*., 2007). When soil moisture content is high, oxygen diffusion rates are limited and this can result in micro-aerobic or anaerobic conditions, particularly in aggregate centres (Arah and Smith, 1990), reducing overall microbial activity (Barros *et al*., 1995) and inhibiting aerobic degradation. Although soil microbial activity is often at its maximum at field capacity (Linn and Doran, 1984), it is interesting to note that rates of triclosan degradation appeared to be enhanced in our study (as evidenced by increased Me-TCS concentrations) at lower soil moisture contents from June onwards – even when approaching wilting point. This may have been promoted by increased soil temperatures during this period which may have increased microbial activity (Eash *et al.,* 2008) and reduced sorption. For most organic pollutants, sorption to organic matter decreases with increasing temperature (ten Hullscher and Cornelissen, 1996; Schwarzenbach *et al*., 1993).

As expected, triclosan leaching, as evidenced by the appearance of triclosan in the 10-20 cm and 20-30 cm layers (Figure 2), appears to have been most active during the winter period (November 2009 to February 2010), when the rate of soil water movement will be enhanced by high soil water content (increased hydraulic conductivity) and, in the study period, by heavy snow fall followed by thawing and heavy rainfall.

Soil pH can also affect the mobility of ionisable organic compounds, such as triclosan, in soil. Neutral triclosan tends to dissociate to form a phenolate anion at high pH and this anion, will be repelled from negatively charged clay particles and from some organic colloids. Anionic triclosan, in addition to being more mobile, is photodegradable (Lyndall et al., 2010) – although this will be of limited relevance in soils. The ratio of anionic to neutral species can be calculated from

$$\log D = \log K_{OW} + \log\left[\frac{1}{1+10^{pH-pKa}}\right]$$

where log *D* is the distribution coefficient (ratio of ionised to un-ionised compound) for a given pH and log $K_{ow}$ is the octanol : water partition coefficient (Thomas and Foster, 2005). The loamy sand soil studied here had an average pH of 8.2 which would result in 53.5% of the triclosan in anionic form at 25°C. This compares with the sandy clay loam and clay soils which had average pH values of 6.6 and 7.1, respectively, resulting in respective ionised fractions of just 2% and 10%. This

means that the mobility of both neutral triclosan and its phenolate ion in the loamy sand soil is likely to be much higher than in the other two soils due to a combination of relatively low organic matter content and relatively high pH. In addition to the loamy sand soil having an increased pH compared to the other soils, it also has a reduced CEC (6.6 cmol+/kg in the loamy sand, 13 cmol+/kg in the sandy loam and 13.5 cmol+/kg in the clay soil), which would reduce the ability of the soil to attract the triclosan, resulting in more movement. Biodegradation rates may also be enhanced because of the lower propensity of the anionic molecule to sorb to soil solids where it may be less accessible to microbes. The expectation of enhanced leaching is corroborated to some extent by the fact that concentrations of triclosan in the 10-20cm layer of the loamy sand soil were higher than in the other two soils (Figure 2) and by the fact that degradation rates (formation of Me-TCS) also appeared to be higher in this soil in all three layers. It is interesting to note that a marked decline in soil pH was observed in the sandy clay loam soil during the study. This could be due to the degradation of sludge which releases $CO_2$, to nitrification of mineralised N (which releases $H^+$ ions) – again from the large volume of sludge applied or to the accumulation of organic acids generated during organic matter degradation (Veeresh, 2003), which can accumulate in small pores (Ngole, 2010). Dewatered sludge has been observed to significantly reduce soil pH due to the acidifying reactions of nitrogen and sulphur oxidation (Richards *et al*., 2000; Harrison *et al*., 1994). In any case, since the pH of this soil is already reasonably low (and, thus, the ionised fraction also low) such a pH change is unlikely to influence mobility or degradation rates.

Overall chemical recovery, triclosan or Me-TCS, was lowest in the clay soil (49% compared to 77% in the sandy clay loam and 84% in the loamy sand). This could be due to a higher rate of mineralisation in this soil, to the formation of undetermined metabolites or to the higher organic matter content of this soil (Table 1) which will increase sorption (Agyin-Birikorang *et al.,* 2010) and reduce both mobility (Behera *et al.,* 2010) and degradation (as evidenced by a lower rate of Me-TCS formation). Fine textured soils generally have slower degradation rates than course sandy textured soils due to greater bioavailability in the coarser soils, especially at sub soil level (Rodríguez-Cruz *et al*., 2006). Higher organic matter content may also affect bound residue formation. Low recovery in the top 30 cm of the clay soil may also be a consequence of different leaching patterns in this soil. It is possible that triclosan advection in the clay matrix may have been limited by a higher fraction of immobile water (van Genuchten and Wierenga, 1977) in the smaller pores of this soil. On the other hand, preferential flow is a common feature of heavy soils, which are more likely to form cracks and macropores. It is, therefore, possible that some triclosan may have been leached beyond the top 30cm of this soil.

Triclosan breakthrough occurred in December 2009 in the 10-20 cm layer and in February 2010 in the 20-30 cm layer in all three soils. Peak triclosan concentrations were observed in in February 2010 (10-20 cm) - probably due to significant snow melt which occurred at this time in combination with heavy rainfall. As in the 0-10 cm layer, the rate of degradation of triclosan in the lower layers tended to increase, in all soils, when soil moisture content fell and temperatures increased. Although the data suggest that most Me-TCS in each layer was formed *in situ*, we cannot exclude

the possibility that there may have been some translocation of Me-TCS from above, via leaching or bioturbation. Degradation rates of organic pollutants often decrease with depth (Soulas and Lagacherie, 2001). This can be attributed in part, to a decrease in organic matter content which will result in decreased microbial biomass implying that there may be less triclosan degradation in the lower depths compared to the top soil. Me-TCS formed in the upper layers of soil may rapidly become bound and unavailable for further degradation (Piutti *et al*., 2002).

The fact that the majority of the chemical extracted from all three soils at the end of the study was in Me-TCS form suggests that of the rate of primary degradation of triclosan was relatively high. This corroborates previous studies which have shown that triclosan can degrade rapidly (Ying *et al*., 2007; Lozano *et al*., 2010), although the formation of Me-TCS was not measured during these studies. The fact that most chemical was still in the upper 10 cm suggests that triclosan is not particularly mobile, again in broad agreement with previous work (e.g. Xu *et al*., 2009).

It is important to recognise that the concentrations of triclosan and Me-TCS reported here were extracted using a relatively harsh extraction process (ASE). Clearly not all of this extracted chemical will be bioavailable (Boethling *et al*., 2009). In addition, it is possible that some parent compound and metabolite was not extracted using the method employed, although the ASE is thought to be one of the most efficient extraction techniques available. In a previous laboratory incubation study using $^{14}C$ triclosan (Butler *et al*., 2010) we observed that only a small fraction of the

radiolabelled sample applied to soil (*ca* 1%) was extractable using $CaCl_2$, which is considered to be the readily available fraction (Gevao *et al*., 2005) and a combined total of approximately 35% of the radiolabel was extractable using a mild solvent extraction system using methanol, representing the potentially available fraction (Mordaunt *et al*., 2005). This suggests that relatively little of the triclosan and Me-TCS measured in the field soils may be bioavailable.

There are no studies to the best of our knowledge showing the degradation of any chloroanisole compound under aerobic conditions. However previous work has shown that chloroanisoles can be degraded under anaerobic conditions in the presence of selected soil microbes such as *Desulfitobacterium frappieri* (Dennie *et al.,* 1998). This could offer some explanation for the apparent persistence of Me-TCS in the soils at the end of the field study. The majority of Me-TCS was formed at a time when the soil was aerobic. However, Me-TCS may be degraded under anaerobic conditions the following winter as observed with 2,4,6-trichloroanisole, which is first O-demethylated before being dehalogenated and ultimately mineralised (Goswami *et al*., 2007).

It is noteworthy that the sludge application rate used in the current study was over 5 times greater than the rate generally used operationally in the UK of 8-10 tonnes per hectare, although usage levels depend on the nutrient content of the soil and in the sewage sludge. An increase in the application rate could have the effect of altering the degradation kinetics in the soil by altering the soil physical and chemical

properties (Weber *et al*., 2007) or by exerting toxic effects on soil microbes. However, the soil concentrations of triclosan observed here were clearly below any expected effect thresholds. Such high applications of biosolids would also have the affect of increasing the concentration of total nitrogen in the soil, which in turn would result in an increase in nitrifying bacteria. Certain nitrifiers have been observed to degrade triclosan, especially in the presence of enzymes produced by ammonia oxidising bacteria, which can function as a catalyst for triclosan degradation in the wastewater treatment facility (Roh *et al*., 2009). However, it is also possible that nitrifiers in the soil convert triclosan into Me-TCS as a defence mechanism against triclosan toxicity. This would need to be confirmed or refuted by further study.

There are a number of irregularities which remain about the mechanisms controlling the fate of triclosan in this experiment. It is curious, for example, that triclosan concentrations in the 20-30 cm layer continued to increase even during the summer months when leaching would not be expected. This could have been due to bioturbation, but further work would be needed to confirm or refute this hypothesis. It is possible that numerical modelling of chemical behaviour could explain the observed phenomena further.

## 4.6 Conclusions

Changes in the concentrations of triclosan and Me-TCS were observed at three different depths in three different agricultural soils over a one year period. Decreases in the concentration of triclosan were due to a combination of

biodegradation, leaching, bioturbation and possibly the formation of non-extractable residues. Further work would need to be undertaken to determine whether triclosan or Me-TCS form non-extractable residues in field soils and whether their formation reduces toxicity due to being non-bioavailable to the soil microbes. All these processes have a strong seasonal component – being driven by changes in temperature, water content and drainage rates. Leaching tended to be restricted to the winter period whereas degradation was more rapid in the summer, as evidenced by the formation of Me-TCS. Chemical behaviour differed between soil types. These differences were consistent with expectations based on soil physical and chemical properties such as organic matter levels, clay content and pH. Triclosan appeared to be more mobile and more rapidly degraded in the lighter soils due to a combination of lower organic matter content and higher pH, which will both tend to increase mobility and the availability of triclosan to micro-organisms. The study shows, for the first time, that most triclosan degradation in field soil results in the formation of Me-TCS, which is more persistent and potentially more bioaccumulative than the parent compound therefore also potentially less bioavailable. This Me-TCS formation is approximately consistent with observations reported in a laboratory incubation study by Waria et al (2011) in which up to 80% of the 14C-labelled triclosan was transformed to Me-TCS in a silty clay loam, although only 20% was recovered as Me-TCS in a fine sand soil.

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# Chapter 5

## Effects of Triclosan on Soil Microbial Respiration

**Butler, E**., Whelan, M.J., Ritz, K., Sakrabani, R., van Egmond, R., 2011. Inhibitory effects of triclosan on soil microbial respiration. *Environmental Toxicology and chemistry, 30,2. 360-366*.

## 5.1 Abstract

The anti-microbial substance triclosan has widespread use in personal-care products and can enter the terrestrial environment if sewage-sludge is applied to soil. The inhibitory effects of triclosan on basal and substrate-induced respiration (SIR) of three different soils were investigated. Soils were dosed and later re-dosed with four nominal triclosan concentrations and respiration rates were measured over time. In each soil there was a significant depression in basal respiration after initial dosing, followed by a recovery. The initial extent of respiration inhibition was positively related to the triclosan dose, i.e., respiration was most inhibited at highest triclosan concentration. Differences in respiration inhibition between soils at equivalent dose were inversely correlated with organic matter and clay content, suggesting that the bioavailability of triclosan may have been reduced by sorption to organic carbon or by physical protection in micro pores. Substrate-induced respiration was also reduced by the addition of triclosan and subsequently recovered. Following re-dosing with triclosan, basal respiration was enhanced in all soils suggesting that it was acting as a substrate. However, re-dosing resulted in SIR inhibition in all treatments above 10 mg triclosan $kg^{-1}$ in all three soils, although all soils appeared to be more resistant to perturbation than following initial dosing. The present study suggests that triclosan inhibits soil respiration but that there is a subsequent acclimation of the microbial community.

**Keywords–**Triclosan, Respiration inhibition, Soil, Resistance, Resilience

## 5.2 Introduction

Triclosan (5-Chloro-2-[2,4-dichloro-phenoxy]-phenol) is a broad spectrum antimicrobial agent and preservative, commonly used in home and personal care products such as toothpastes, detergents, soaps, cosmetics, deodorants, and mouthwashes, as well as being incorporated into polymers and fibres to make clothes, shoes, food chopping boards, and sportswear (McAvoy, *et al.*, 2002; Capdevielle *et al*., 2008). It has been estimated that over 350 tonnes of triclosan are produced annually for European use alone (Singer *et al*., 2002). In many of its applications, triclosan is rinsed down the drain during normal usage, which can result in environmental exposure (McAvoy, *et al.*, 2002). In the UK, triclosan consumption in products which result in "down-the-drain" emission after use was estimated to be approximately 1 g capita$^{-1}$ yr$^{-1}$ in 2007 (Capdevielle *et al*., 2008).

Triclosan is moderately soluble in water at neutral pH (12 mg l$^{-1}$) and has a dissociation constant (p$K_a$) of 8.14. It is also relatively non-volatile with a vapour pressure of 7 x 10$^{-4}$ Pa at 25ºC (Reiss, 2009) and a Henry's law constant (ratio of vapour pressure to aqueous solubility) of 1.28 × 10$^{-2}$ Pa m$^3$ mol$^{-1}$. Triclosan is also hydrophobic with a log octanol: water partition coefficient (log $K_{OW}$) of 4.8 (Reiss, 2009). This suggests that triclosan will sorb to organic matter, resulting in the likelihood of high triclosan concentrations in sewage sludge (Heidler and Halden, 2007; Ying and Kookana, 2007; Kinney *et al*., 2008). Sludge is increasingly being used as an additive on agricultural land (Schowanek *et al*., 2007) providing a significant route for soil exposure to chemicals such as triclosan (Kannan *et al*., 2007; Heidler *et al*., 2006).

The fate and effects of triclosan in aquatic environments have been well studied (Capdevielle *et al*., 2008; Singer *et al*., 2002; Reiss *et al*., 2002; Lindström *et al.,* 2002; Wilson *et al*., 2008) and attention in the recent literature has started to examine the effects (Reiss, 2009; Waller and Kookana, 2009), degradation (Ying and Kookana, 2007) and transport (Lapen *et al*., 2008; Topp *et al.,* 2008; Xu *et al*., 2009; Langdon *et al*., 2010) of triclosan in soil. Since triclosan is known to have antimicrobial properties, it is feasible that it will have some inhibitory effects on soil microbial functions, such as respiration and nitrification. Although Ying and Kookana (2007) failed to observe respiration inhibition over 70 days in sand and clay soils dosed with 1 mg triclosan $kg^{-1}$ soil, they did note a significant reduction in dehydrogenase activity. Waller and Kookana (2009) reported enhanced soil respiration in a sandy soil but respiration inhibition in a clay soil dosed at 10 mg triclosan $kg^{-1}$. The clay soil had higher organic carbon content than the sandy soil, which would imply reduced bioavailability of triclosan with an associated reduction in expected toxicity. However, inherent differences in the soil microbial communities of the two soils will also affect their responses to triclosan addition. Addition of triclosan inhibited nitrification in both soil types.

In the present study, we report observations of the effects of triclosan dosing on microbial respiration and biomass in three contrasting soils from Eastern England. Following initial application, soils were re-dosed with triclosan to determine the extent to which the soil microbial community had adapted to this initial exposure. It is hypothesised that triclosan dosing will have an inhibitory effect on both microbial

respiration and biomass, but that the soils will show some acclimation to triclosan after re-dosing.

## 5.3 Methods

### 5.3.1 Soil sampling

Three different soils (a sandy loam, clay and loamy sand) were collected from Silsoe farm in Bedfordshire, UK (26˚25'W, 52˚26'N; 25˚56'W, 52˚12'N and 26˚27'W, 52˚33'N respectively). All samples were taken from the upper 20 cm of agricultural fields which have been under an arable rotation dominated by winter wheat and peas for over 20 years. Samples were refrigerated at 5°C immediately after collection and transported to the laboratory. Soil used in the experiment was sieved moist to ≤ 2mm, manually mixed and air-dried following standard methods BS 7755 Section 2.6: 1994 and BS ISO 11464 2006 respectively. Basic properties of each sieved soil were analysed. Soil pH was determined following BS ISO 10390:2005; Total organic matter was obtained via loss on ignition (BS EN 13039: 2000); Total carbon and total nitrogen content was calculated after elemental analysis (BS 7755 section 3.8 and BS EN 13654-2 2001, respectively). Particle size distribution, electrical conductivity and cation exchange capacity were all obtained following BS 7755 methods (sections 5.4:1998, 5.5: 1999, 3.4: 1995 and 3.12: 1996 respectively). All results are shown in Table 5-1.

**Table 5-1:** Basic properties of the three soils used in the experiments

| Property | Loamy sand | Clay | Sandy loam |
|---|---|---|---|
| pH | 7.5 | 7.1 | 6.9 |
| EC µS/cm | 72.8 | 55.9 | 82.7 |
| CEC cmol+/kg | 6.6 | 13.0 | 13.5 |
| TOM (% by mass) | 4.0 | 7.6 | 5.9 |
| TC (% by mass) | 1.7 | 2.8 | 2.3 |
| Sand % | 71.9 | 19.4 | 63.7 |
| Silt % | 16.4 | 20.2 | 18.9 |
| Clay % | 11.7 | 62.1 | 17.4 |
| Total nitrogen % | 0.2 | 0.3 | 0.2 |
| WHC (% $m^3m^{-3}$) | 43.4 | 74.5 | 58.0 |

**EC – electrical conductivity, CEC – Cation exchange capacity, TOM – Total organic matter, TC – total carbon, WHC- water holding capacity.**

### 5.3.2 Soil microcosm preparation

Each soil was dosed in laboratory microcosms at five different nominal triclosan concentrations: 0, 10, 100, 500 and 1000 mg $kg^{-1}$ (dry mass). Although the use of nominal concentrations can introduce significant uncertainties into toxicity studies, we feel that it is defensible here because the primary goal of the work was to understand the stressor-response relationship rather to determine toxicity end points, such as EC50s. It should be noted that the data generated here is not appropriate for deriving such end-points because we expect triclosan concentrations and availability to change over the course of the experiment. It should also be noted that the nominal concentrations used in the study are relatively high compared with expected triclosan concentrations in soils amended with sewage sludge (e.g. 69-833 µg $kg^{-1}$) (Kinney *et al*., 2008), although concentrations as high as 55 mg $kg^{-1}$ have been reported in sewage sludge itself (Heidler and Halden, 2007). Each treatment was repeated in triplicate, making a total of 45 microcosms (15 for each soil type).

Each microcosm consisted of a 200 ml glass screw-top jar containing 50 g dry mass of soil, amended to 60 % water holding capacity and incubated at 20˚C for 14 days prior to addition of triclosan (day zero) to allow the soil microbial community to adjust to the incubation temperature. Dosing stock solutions with different triclosan concentrations were made up by dissolving powdered triclosan (Wellington Labs, USA) in ethanol. On day zero, 1 ml of the appropriate stock solution was added to each jar, 1 ml of ethanol was added to each of the controls. All soils were subsequently re-spiked to the same nominal concentrations on day 54. For each dosing operation, the triclosan was thoroughly incorporated by manual mixing and the jars were left open for 1h in a fume cupboard to allow the ethanol to evaporate. The jars were then closed and incubated at 25˚C and sampled after 0,1,2,3,4,5,6, 14 and 54 days following initial dosing. The soils were also sampled on days 54, 55, 56, 57, 58, 59, 60, 61 and 68 (i.e. post redosing). The jars were vented daily to keep the systems aerobic.

### 5.3.3 Microbial Respiration

Microbial respiration was measured using an automated multi-channel respirometer based upon the Rapid Automated Bacterial Impedance Technique (RABIT: Don Whitley Scientific, Shipley, UK). The system operates by measuring changes in the electrical conductivity of an alkaline agar which traps respired $CO_2$ (Haigh and Rennie, 1994; Ritz *et al*., 2006). Basal respiration is a measure of the total metabolic activity of microorganisms, soil fauna and roots in a soil sample. In this experiment, distinction was not made between the contributors to $CO_2$ emissions and the term basal respiration was used to describe the gross $CO_2$ evolution from

soil. Substrate-induced respiration (SIR) is a measure of the $CO_2$ evolved from a soil sample after administering an optimal concentration of an additional energy source, and is directly proportional to the soil microbial biomass (Anderson and Domsch, 1978).

On each sampling occasion, the soil in each microcosm was manually mixed and two 1 g aliquots were weighed into glass boats. Deionised water was added to one aliquot by pipette to bring the soil up to 100% water holding capacity for measuring basal respiration. Glucose solution (200 mM) was added to the second aliquot, for SIR determination. As the soil was maintained at 60% water holding capacity (WHC), the amount of water and glucose added varied between the sand, loam and clay soils (170, 230 and 300 µl glucose $g^{-1}$ soil respectively resulting in nominal glucose concentrations of 6, 8.3 and 11 mg $g^{-1}$). The RABIT cells were prepared as follows: One g of bacterial agar was added to 50 ml deionized water in 100 ml screw-capped glass bottles and dissolved by autoclaving. A 10% stock solution of KOH was prepared (25 g KOH in 250 ml of deionised water) from which a 1% KOH working solution was made on the day of use. Whilst the agar was still hot, 50 ml of the 1% KOH solution was added and thoroughly mixed. One ml of the agar mixture was then pipetted into the base of each RABIT cell. The agar was allowed to cool and solidify for approximately 15 minutes before applying rubber stoppers to seal the tube. Each cell was allowed to stabilise for a minimum of 3 h at room temperature before use. The glass boats containing the soil samples plus the water or glucose solutions were then placed in respirometer cells which were then resealed with rubber bungs before inserting into the respirometer in a random arrangement to

eliminate any systematic bias. The change in conductivity was measured every 6 minutes over 16 hours. These data were then converted to equivalent $CO_2$-C evolved per unit dry mass of soil per hour (µg $CO_2$-C $g^{-1}$ $h^{-1}$) using a factor derived from a calibration fit which compared respiration rates obtained using the RABIT technique to a reference method using headspace gas chromatography (Haigh and Rennie, 1994).

In all cases, respiration over the first two hours was assumed to be lag phase and was discarded (Carter and Gregorich, 2007). The basal respiration rate was calculated as the mean rate of $CO_2$ evolution between 2 and 16 hours. For SIR, the respiration rate between 2 and 6 hours was used to calculate the microbial biomass (Anderson and Domsch, 1973.

### 5.3.4 Statistical analysis

Results are presented as means of three replicates per treatment +/- the standard error of the mean. Statistica (version 9) was used to perform all statistical analysis. Daily between-treatment variations were analysed using a two-way factorial analysis of variance (ANOVA) to determine any effects of soil type and triclosan dosing. Temporal patterns were analyzed using repeated measures ANOVA, where the dependant variables are the respiration rates taken daily and the independent variables are the nominal triclosan concentration (dosing concentration) and the soil type. Fisher's least significant difference (LSD) test with an alpha value of 0.05 was

used to determine statistically significant treatment effects for any significant ANOVA results.

## 5.4 Results

### 5.4.1 Initial response to triclosan addition

There was a clear dosing effect for each soil (i.e. depression of SIR and basal respiration rate on the day after initial spiking with increasing nominal concentration of triclosan) which was statistically significant in many cases (Factorial ANOVA with Fisher's LSD test, $p<0.05$). The treatment effect on basal respiration on the day following dosing (Figure 1) was most pronounced in the loamy sand and the clay soils. In both of these soils, the depression in basal respiration with increasing triclosan concentration was curvilinear with a marked decrease in respiration even at the lowest triclosan dose rates and an increasing depression with an increase in triclosan dose (Figure 1). The effect of triclosan on basal respiration in the sandy loam soil was weaker, although all treatments receiving triclosan had significantly lower basal respiration rates compared with the control ($p<0.05$).

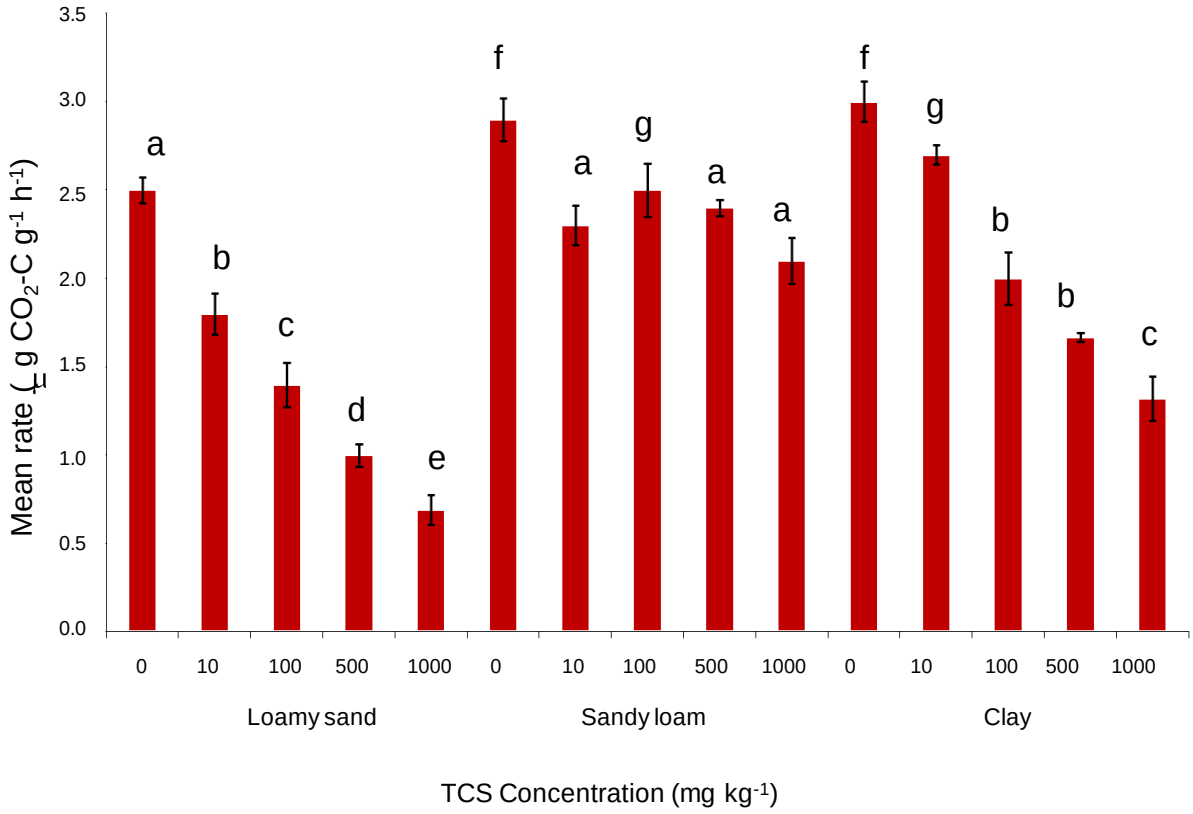


**Figure 5-1: Mean Basal respiration rate for each treatment and each soil measured between 2-16 hours on the day after initial spiking. Error bars show the mean + one standard error (se) of the mean. Factorial ANOVA with Fishers LS test produced letters a-f, showing significant between soil and between triclosan concentration treatment differences at $p < 0.01$. Soils listed in order of increasing clay content and organic matter.**

Treatment effects on SIR on the day following initial dosing (Figure 2) were, again, most pronounced in the loamy sand soil and, to a lesser extent, in the clay soil and least pronounced in the sandy loam. In general, the clay and sandy loam had higher

average absolute SIR rates, for any given treatment, including the controls, than the loamy sand.

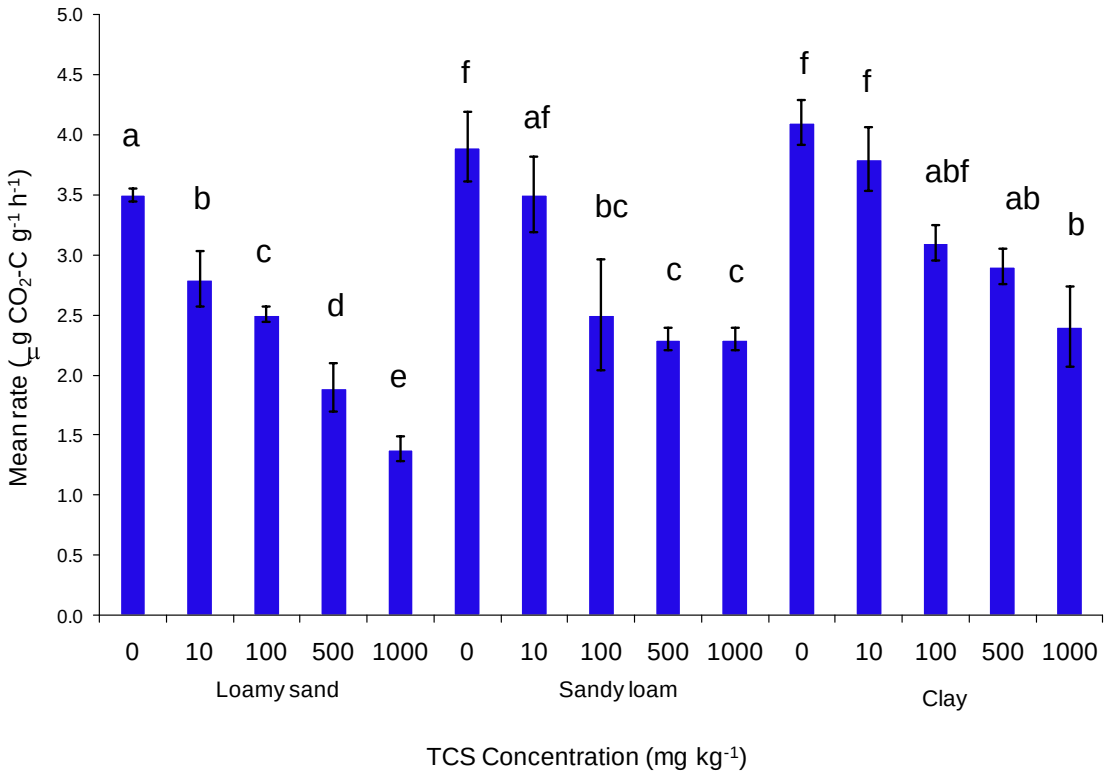


**Figure 5-2:** Mean SIR rate for each treatment and each soil measured between 2-6 hours on the day after initial spiking. Error bars show the mean + one standard error (se) of the mean. Factorial ANOVA with Fishers LS test produced letters a-f, showing significant between soil and between triclosan concentration treatment differences at $p < 0.01$. Soils listed in order of increasing clay content and organic matter.

### 5.4.2 Change in basal respiration after initial spiking

Figure 3 highlights the temporal change in basal respiration rate over the first 14 days after initial spiking relative to the respective control. In all three soils, there was

an initial depression of basal respiration followed by a recovery. In the loamy sand (Figure 3a), basal respiration declined in the first two to four days after spiking at all triclosan dose rates and then recovered. In the sandy loam (Figure 3b), the decline in basal respiration was similar to that in the loamy sand but recovery was more rapid. In the case of the clay soil (Figure 3c); respiration inhibition was generally less pronounced than in the other two soils. In all three soils, the initial depression was most apparent for the treatment with the highest triclosan dose rate (1000 mg $kg^{-1}$) and least apparent for the 10 mg $kg^{-1}$ treatment. Maximum depression was between 50 and 70% of the control rate in the soil spiked at 1000 mg $kg^{-1}$.

Four days following dosing, basal respiration rates in the sandy loam (Figure 3b) treated with 10 and 100 mg triclosan $kg^{-1}$ were equal to or higher than those in the control. The recovery was slower in higher dose treatments. Complete recovery was also apparent in the clay soil after day 5 for the 10 mg $kg^{-1}$ treatment and after about day 7 for the other treatments. Basal respiration for the 10 mg $kg^{-1}$ treatment in the loamy sand soil remained lower than in the control for the whole two weeks following initial spiking.

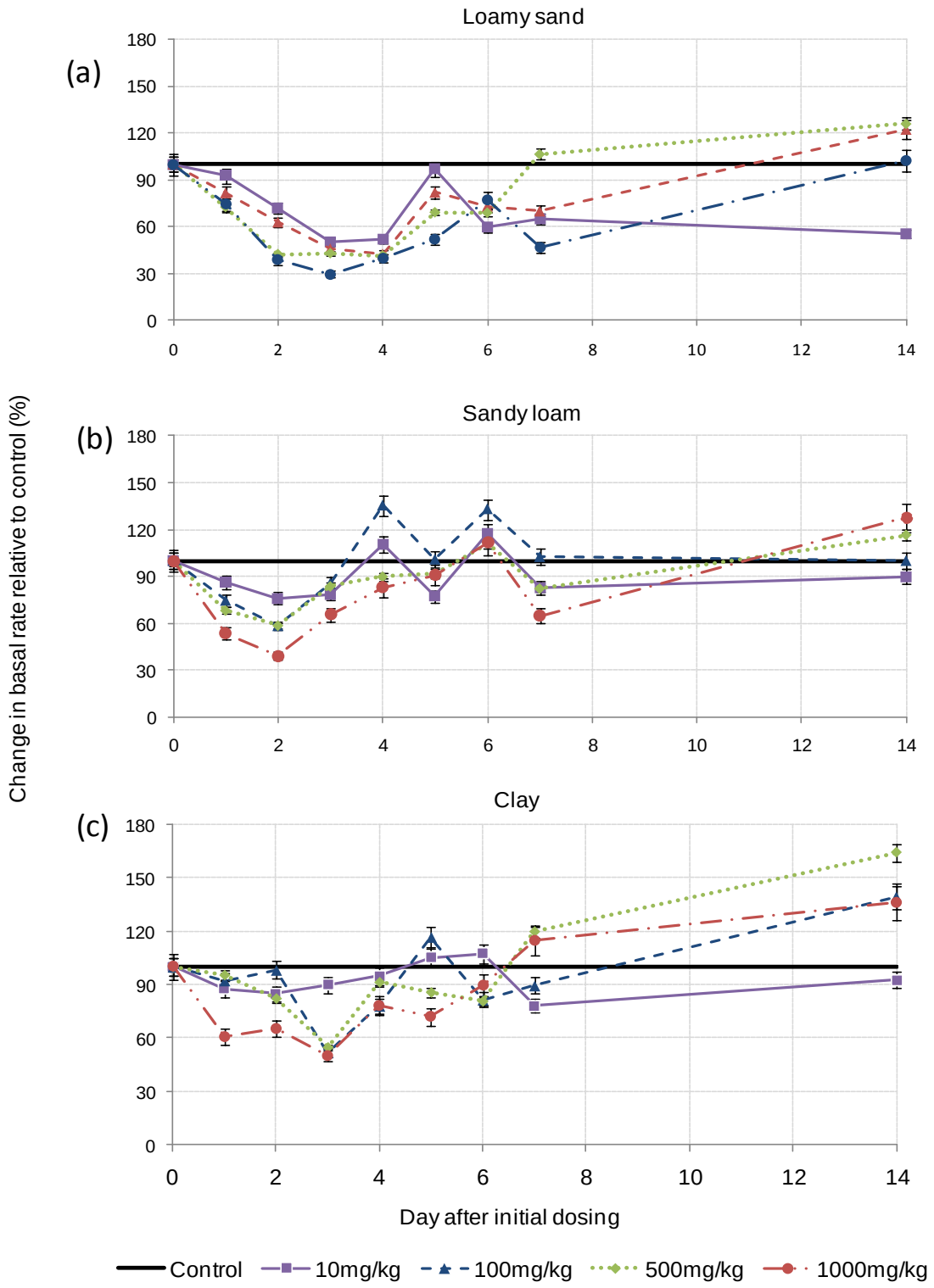


**Figure 5-3: Changes in basal respiration in each soil over the first 14 days after initial spiking. (a) Sandy loam; (b) Loamy sand; (c) Clay. Rates are plotted as a percentage of those observed in the control (0 mg triclosan $kg^{-1}$).**

### 5.4.3 Change in SIR after initial spiking

Changes in SIR for each soil and each treatment over the first 14 days after initial spiking with triclosan are shown in Figure 4. Overall, the temporal pattern was very similar to the temporal pattern observed in basal respiration. In all three soils, for most treatments, there was a depression in SIR followed by recovery. The depression was most pronounced in the case of soils treated with 1000 mg $kg^{-1}$ and least pronounced in the soils treated with 10 mg $kg^{-1}$. Some stimulation of SIR was apparent in the case of the 10 and 100 mg $kg^{-1}$ treatments in the loamy sand soil but by day 2, the SIR for these treatments was lower than that in the control soil. In all three soils, SIR was equal to or greater than the control SIR by day 7 in most treatments. In the case of both the loamy sand and the clay soils, SIR was significantly greater than that in the control on day 14 of the experiment in all treatments. Many of the SIR rates in all soils were significantly different from the control (repeated measures ANOVA, $p<0.05$).

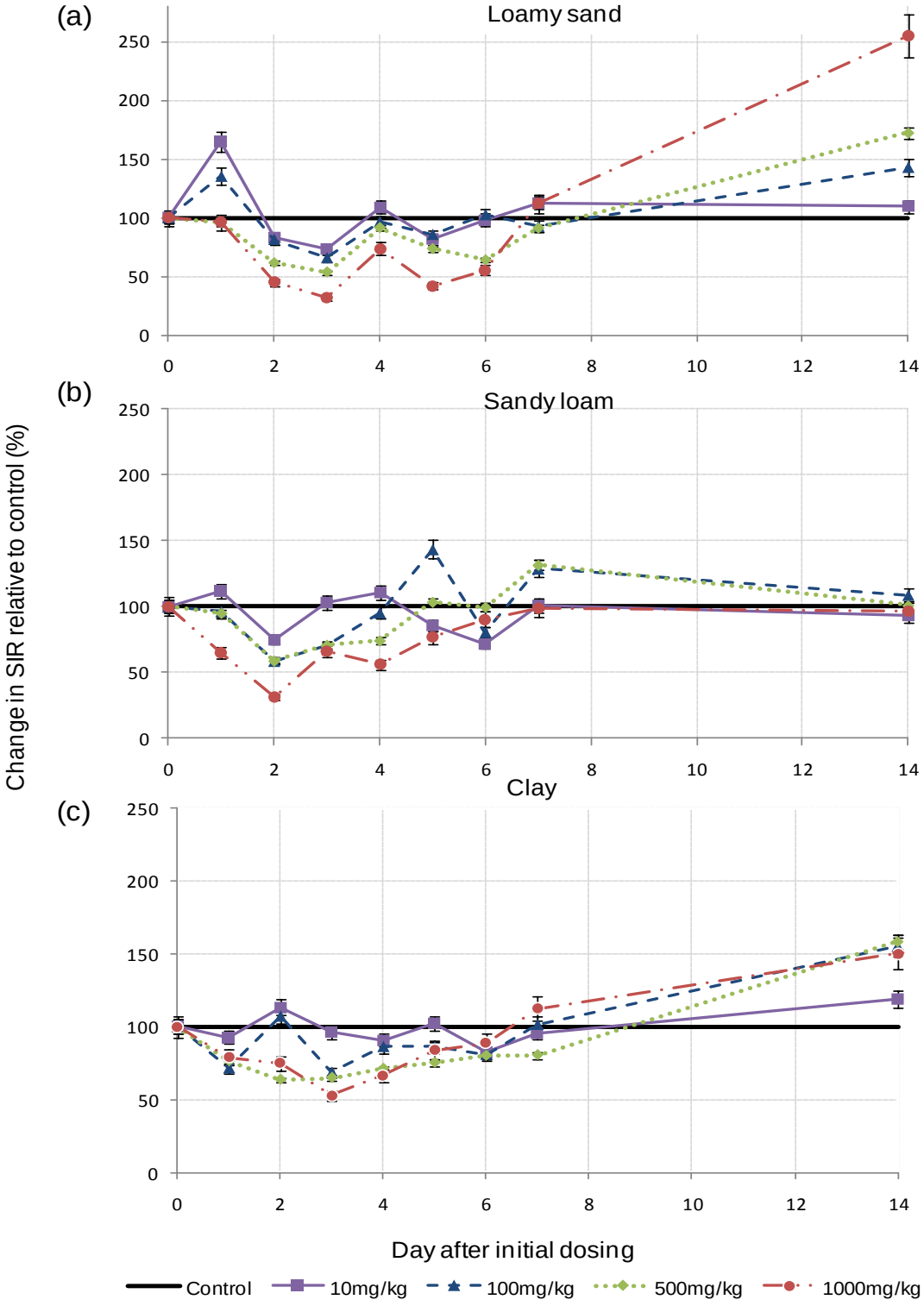


**Figure 5-4: Changes in SIR in each soil over the first 14 days after initial spiking. (a) Sandy loam; (b) Loamy sand; (c) Clay. Rates are plotted as a percentage of those observed in the control (0 mg triclosan kg$^{-1}$).**

### 5.4.4 Basal respiration changes following re-dosing

Changes in basal respiration following re-dosing soils with triclosan are shown in Figure 5-5. The temporal pattern observed for all three soils was quite different to that observed following initial dosing (Figure 5-3). In most cases, there appeared to be a stimulation of respiration. However, by day 14 after re-dosing (i.e. day 68 after initial dosing), basal respiration in the loamy sand and the clay soils had returned to levels which were similar to those observed immediately prior to re-spiking (i.e. on day 54 of the whole experiment), with the exception of the 10 mg $kg^{-1}$ treatment in both soils. Apart from the 1000 mg $kg^{-1}$ treatment, basal respiration in the sandy loam soil (Figure 5-5b) remained higher than in the control at the end of the experiment.

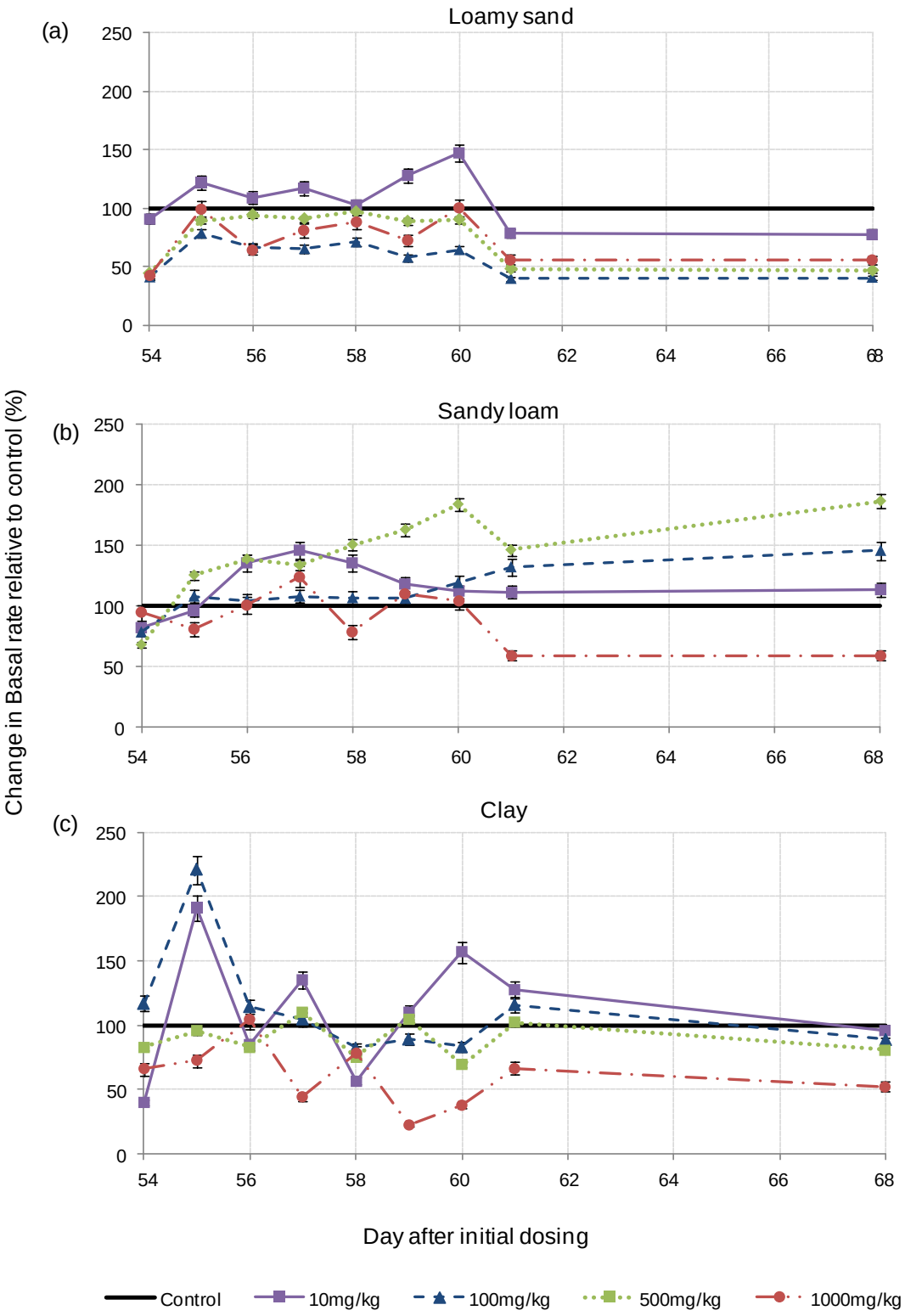


**Figure 5-5: Changes in basal respiration in each soil over 14 days following re-spiking with triclosan. (a) Sandy loam; (b) Loamy sand; (c) Clay. Rates are plotted as a percentage of those observed in the control (0 mg triclosan kg$^{-1}$).**

### 5.4.5 Substrate induced respiration changes following re-dosing

The change in SIR after re-dosing is shown in Figure 5-6. For the loamy sand soil, SIR following re-dosing was at or below the SIR in the control soil for the whole period following re-dosing. Although there was a depression in SIR following triclosan re-dosing (day 1 in Figure 5-6a), SIR appeared to recover to some extent in the 10 mg $kg^{-1}$ treatment. The SIR response in the clay soil (Figure 5-6c) was more pronounced with a decrease in SIR over the first four days after re-dosing in most treatments, followed by a marked recovery. By day 6, the SIR for all treatments was equal to or greater than the SIR in the control soil. In the sandy loam soil the temporal pattern in SIR was rather different. In some treatments there was a depression in SIR following re-dosing, which was most apparent on day 2. However, SIR increased in all treatments thereafter and by day 4 exceeded SIR in the control soil for the 10 mg $kg^{-1}$ and 500 mg $kg^{-1}$ treatments. By day 7 after re-dosing, SIR was greater than the control SIR in all microcosms except for the 100 mg $kg^{-1}$ treatment.

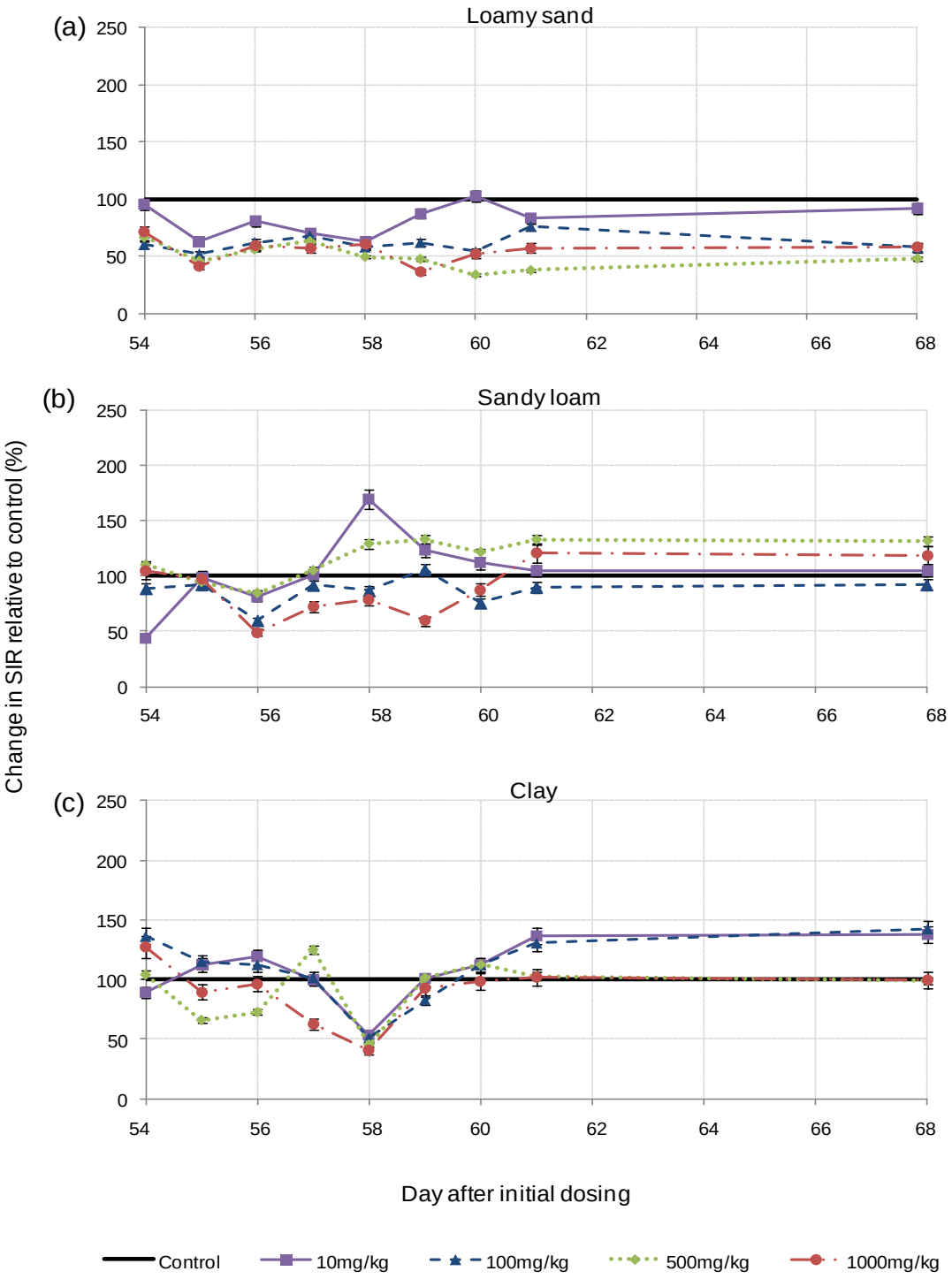


**Figure 5-6: Changes in substrate induced respiration in each soil over 14 days following re-spiking with triclosan. (a) Sandy loam; (b) Loamy sand; (c) Clay. Rates are plotted as a percentage of those observed in the control (0 mg triclosan $kg^{-1}$).**

### 5.4.6 Effect of soil properties

A general linear model (GLM) was fitted to the respiration data using the nominal triclosan concentration as a categorical predictor and clay content, pH and organic matter content as continuous predictors to determine a statistical relationship between these soil properties and the SIR and basal respiration inhibition induced by the initial dosing. The GLM showed that clay content, organic matter content and pH statistically affect the respiration response, independent of triclosan dose ($p$<0.05). Triclosan dose was identified as the main contributor to the observed variability in respiration response. The soil physical properties affected the respiration responses to different extents in SIR and basal respiration. Excluding dose, pH contributed to 21% of the observed variability in basal respiration but only accounted for 1% of the variation in SIR response. Clay and organic matter content contributes towards 12% and 8% of the variability in basal respiration, respectively and towards 16% and 15% of the variability, respectively, in SIR response.

## 5.5 Discussion

The soils examined in this study exhibited classical ecological responses to perturbation (Orwin and Wardle, 2004; Pimm, 1984). Triclosan inhibited both basal and substrate-induced respiration when added to non-acclimated soils. The stimulation of respiration followed by a return to baseline implies that the triclosan which was added on re-dosing was being utilised as a substrate. The fact that the most pronounced “stimulation” of respiration appeared to be observed for the low dose rates (10 and 100 mg $kg^{-1}$) suggests that there may have been an interaction between substrate utilisation and toxic effects i.e. that the re-dosed triclosan was

acting as a substrate at low concentrations but was having toxic effects at high concentrations. Although basal respiration appeared to be stimulated by triclosan after re-dosing, the general pattern of a contemporaneous decline in SIR implies that the microbial biomass was affected overall. This tends to confirm the hypothesis that triclosan acts as both a substrate and a toxin to the microbial community after acclimation to the initial dose. The observed enhancement in basal respiration may have been promoted by a relatively small fraction of the soil microbial community which was resistant to triclosan toxicity and was able to utilize the energy in the added triclosan.

Inhibition was most pronounced in the loamy sand soil, which could have been due to a lower concentration of organic matter in this soil compared with the other two soils, resulting in a lower sorbed fraction and an increase in triclosan bioavailability. Respiration inhibition was generally lower in the clay soil compared with the other two soils. This could have been the result of reduced bioavailability caused by triclosan diffusion into the smallest pores, which are inaccessible to microbes (Hamscher et al., 2002; Semple et al., 2003) as well as sorption to organic matter. Enhanced bioavailability has also been used in the interpretation of pesticide and antibiotic inhibition of microbial activity and microbial biomass (Thiele-Bruhn and Beck, 2005) although it is pertinent to note that Waller and Kookana (2009) reported triclosan-induced respiration inhibition in a clay soil but not in a sandy soil. Another potential reason for the higher degree of respiration inhibition in the loamy sand is the higher pH in this soil. As pH increases, more triclosan will be present as the phenolate anion, which is more mobile and, therefore, potentially more bioavailable

to the soil microbes. Although most triclosan exists predominantly in the neutral phenolic form in all the soils studied here, the fraction of triclosan in the phenolate anion form in the loamy sand (pH of 7.5) would be higher at approximately 18% compared with approximately 7% in the sandy loam and clay soils (pH 6.9 and 7.1, respectively).

Basal respiration was similar to or greater than that in the control by day 14 of the experiment in at least some of the treatments for each soil, but this was not maintained (compare rates on day 14 in Figure 3 with rates on day 54 in Figure 5). Substrate induced respiration rates generally recovered after triclosan dosing and in some cases, particularly in the sandy loam, exceeded those of the control by day 14, although rates appeared to return to levels similar to those in the control just before re-dosing. It is not currently known whether or not the changes in microbial biomass inferred by changes in SIR, resulted in changes in community structure. Although an attempt was made to investigate this using phospholipid fatty-acid (PLFA) profiles (Calbrix *et al*., 2005), the analysis was not possible due to interference from residual triclosan in the fatty acid chromatograms.

Most soil micro-organisms are dormant (Jenkinson and Ladd, 1981) which explains why basal respiration is generally low compared to SIR. The stimulation of basal respiration observed following triclosan re-dosing suggests that triclosan was being utilised as a substrate by microbes which are both resistant to triclosan toxicity and which are able to synthesise appropriate enzymes for triclosan degradation.

Alternatively, the respiration stimulation could have been stress-induced (Schipper and Lee, 2004) or may have resulted, in part, from the mineralization of a toxicity-enhanced microbial "necromass" by remaining microbes (Hendrickson, 1985; Vaclavik et al., 2004). The response of SIR to re-dosing is less clear and suggests that total microbial biomass may be reduced in some cases following re-dosing. It is likely that some organisms will be more sensitive than others to triclosan, which could affect the activity of the remaining taxa (Meade *et al.,* 2001).

The results of this study can be looked at in the context of ecological stability i.e. resistance (tolerance) and resilience to perturbation (Orwin and Wardle, 2004; Pimm, 1984), where resistance is inversely proportional to the extent of the change caused by a perturbation and resilience is the rate at which a system recovers to its pre-disturbance level (Pimm, 1984). In these terms, the extent of respiration inhibition as a response to triclosan addition is related to the resistance of the soil microbial community and resilience is expressed as the rate at which the respiration inhibition recovers to levels greater than or equal to those of the control. Inhibition of both microbial activity (basal respiration) and biomass (SIR) was generally lowest in the clay soil and the rate of recovery was also fastest in this soil. Although this could reflect a higher degree of microbial resistance and resilience, the results of the GLM (relating respiration inhibition to clay content, organic matter and pH) suggest that this may also be due to lower bioavailability of triclosan in this soil compared with the others.

The findings reported in this study are in broad agreement with data reported by others demonstrating significant short-term inhibition of heterotrophic activity (respiration) and biomass (SIR) at high triclosan concentrations followed by recovery (Ying and Kookana, 2007; Marks *et al*., 1999; Liu *et al.,* 2009). It should be noted that although the lowest two triclosan dose rates (10 and 100 mg $kg^{-1}$) used in this study were comparable to expected concentrations in sewage sludge (e.g. 16 and 55 mg $kg^{-1}$ were reported by Ying and Kookana (2007) and by Heidler and Halden (2007), respectively), they were relatively high for soil and are likely to greatly exceed typical triclosan concentrations in field soils receiving sewage sludge. This limits their relevance for determining meaningful ecological effect thresholds for risk assessment purposes. However, the high dose rates do provide an effective means of perturbing the soil microbial community and, as such, provide a way of evaluating the resistance and resilience of important soil functions. A key outstanding question is whether the differences in resistance and resilience observed in the different soils investigated here reflect differences in the composition of the soil microbial communities or simply differences in triclosan bioavailability.

## 5.6 Conclusions

Triclosan was shown to inhibit respiration and to depress microbial biomass in three different soils. The extent of respiration inhibition was related to the triclosan dosing rate. Both basal respiration and SIR recovered from the initial perturbation to levels which were similar to or greater that those observed in control soils. Furthermore, re-dosing the soil with triclosan resulted in a lower inhibition of soil function compared with the response to initial dosing. In some cases microbial functions

appeared to be stimulated by the re-addition of triclosan. This demonstrates resilience of the soil microbial community to this type of perturbation and, in addition, suggests that the soil microbial community can acclimate to triclosan exposure. Soil microbes with high tolerance to triclosan and or the ability to utilize triclosan as a carbon source would be at an advantage following triclosan application and may be more prevalent – at least in the short term. The results show that the degree of respiration inhibition inversely correlates with soil organic matter and clay content, implying reduced bioavailability. Soil pH control on the ratio of neutral to anionic triclosan may also have played a role in regulating neutral triclosan bioavailability and consequent toxicity. The results presented here suggest low-level triclosan exposure may not have a significant long term impact on the microbial communities of field soils receiving sewage sludge. However, further work is required to confirm this hypothesis.

# Chapter 6

## Solvent-based washing to remove contaminant carryover in soil extracts for phospholipid fatty acid analysis

**Butler, E**., Whelan, M.J., Ritz, K., Sakrabani, R., van Egmond, R., 2011. Solvent-based washing removes lipophilic contaminant interference with phospholipid fatty acid analysis of soil communities. *Soil biology & Biochemistry*, 43, 2208-2212.

## 6.1 Abstract

Phospholipid fatty-acid (PLFA) analysis is an informative method for characterising and quantifying changes in the phenotypic profile of the soil microbial community when soils are exposed to chemical toxicants and other xenobiotics. However, where such materials are lipophilic, a range of non-polar compounds can be co-extracted with PLFAs and can consequently mask PLFA chromatograms. We found this to be the case with the lipophilic, anti-microbial compound triclosan, which can enter the soil via the addition of sewage sludge. A simple method of washing soil in solvent prior to extraction was developed in order to remove triclosan without altering the relative abundance of PLFAs. Three contrasting soils were spiked with 500 mg $kg^{-1}$ of triclosan before being washed with methanol (MeOH), dichloromethane (DCM), hexane or aqueous solutions of these solvents. PLFAs were then extracted and analysed. All treatments were found to remove triclosan effectively, allowing all peaks to be identifiable. However, whilst the polar solvents MeOH and DCM significantly altered the relative abundance of extracted fatty acids in most of the soils tested, soil washing with a small quantity of hexane was able to remove triclosan whilst best preserving the fidelity of the PLFA profiles.

**Key Words:** Contaminant, PLFA, triclosan, soil washing

## 6.2 Introduction

Soils can be exposed to a wide range of contaminants and organic chemicals through a variety of pathways including atmospheric deposition, spillages and via the application of sewage sludge as a fertiliser (e.g. Kannan *et al*., 2007, Heidler *et al*., 2006, Schowanek *et al*., 2007). However, knowledge of the terrestrial fate and effects of many organic chemical contaminants is frequently limited compared with equivalent understanding for aquatic systems. Phospholipid fatty acids (PLFAs) are present in the membranes of all living cells but are easily decomposed by hydrolytic cellular enzymes after cell death, ensuring that the primary source is living cells. The spectrum of PLFAs extracted from a soil provides a phenotypic description of the associated soil community, reflecting species composition and abundance (Findlay, 2004). It has been extensively used to track both spatial (e.g. Bach *et al,* 2010; Mele and Crowley, 2008; Ritz *et al*, 2004) and temporal (e.g. Moore-Kucera and Dick, 2008; Hamel *et al*, 2006) changes in community structure. The technique has been applied to determine the effects of pollutants on soil communities, including metals (e.g. Farrell *et al., 2010;* Sverdrup *et al*., 2006; Tischer *et al*., 2008) and organic toxicants (e.g. Elsgaard *et al*., 2001; Bartling et al., 2009; Zhang *et al*., 2010). One potential problem with PLFA profiling in studies where lipophilic chemical toxicants have entered the soil is that the solvent-based extraction procedures for PLFAs (Frostegård *et al.,* 1991; Bligh and Dyer, 1952; White *et al.*, 1979) can co-extract such toxicants. If these concentrations are high relative to the concentrations of extracted PLFAs, this can result in carryover into chromatogram peaks which can mask the PLFA profile. We observed this to be the case with respect to the anti-microbial compound triclosan (Fig. 1), which has been shown to affect soil microbial respiration (Butler *et al*, 2011). In this paper, we describe the evaluation of a simple

and effective method to remove a representative lipophilic compound from the soil prior to PLFA extraction without modifying the apparent phenotypic profile.

Although triclosan concentrations in soil are relatively low, the concentrations observed in sewage sludge are much higher. Triclosan has also been seen to bioaccumulate in some matrices therefore it is relevant to consider the effects of this compound at all concentrations. Triclosan was seen to co-elute with extracted phospholipids at concentrations of 100 mg $kg^{-1}$ and above so for the purpose of this method development we chose to work at a triclosan concentration of 500 mg $kg^{-1}$ to test the validity of the method as other lipophilic compounds could be found in higher concentrations.

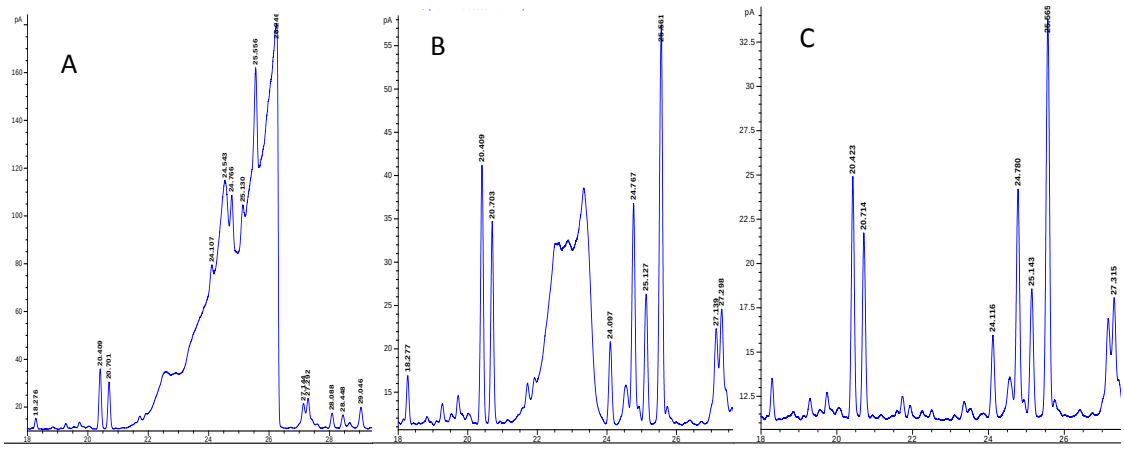


**Figure 6-1:** Examples of a PLFA profile A) with Triclosan (1000 mg $kg^{-1}$) B) with triclosan (500 mg $kg^{-1}$) and C) Same soil type without triclosan addition.

## 6.3 Materials and methods

Samples were taken from the upper 20 cm of three contrasting agricultural soils (Table 1) (a sandy loam, a clay and a loamy sand) co-located at Silsoe farm

(Bedfordshire, UK). Soils were sieved whilst moist to < 2 mm and stored at 4ºC. Aliquots of soil (10 g) were placed into 50 ml glass vials and three treatments were established in triplicate: (i) controls; (ii) spiked with triclosan; (iii) spiked with triclosan and washed with one of six solvent combinations (20 mL). These were hexane, dichloromethane (DCM) and methanol (MeOH), and aqueous solutions of these solvents, containing 50% solvent: deionised water by volume. This made one phase extractants with the more polar MeOH and DCM solvents but produced a two phase extractor with the non-polar hexane solvent. The triclosan was dissolved in ethanol and 100 µL was added to produce a triclosan concentration of 500 mg $kg^{-1}$ dry soil. All soils were left for 48 hours before further treatment to allow the system to recover from the disturbance caused by the preparation process. Although the addition of triclosan is likely to have influenced the soil microbial community relative to the controls, the PLFAs from dead cells are not assumed to decompose over this 48 hour period (Drenovsky *et al*, 2004). The appropriate solvents were then added and all vials were shaken side-to-side for two hours at 300 rpm, centrifuged at RCF 700 g for 5 minutes and the aqueous phase decanted using a Pasteur pipette. The soils were then left to dry overnight at 37˚C, frozen at -80˚C for a minimum of 24 hours and freeze-dried. It is possible that this drying step exceeds the maximum temperature for the mesophilic bacterial community causing cell death. However the rapid freezing and freeze drying of the soils should preserve the membrane structures adequately enough to not affect the PLFA profiles. PLFA analysis was conducted following the method of Frostegård *et al.* (1993). Separated FAMEs were then compared with known retention times to identify individual PLFAs using a Supelco 26 PLFA peak standard (Sigma Aldrich, Dorset, UK). The effects of soil washing on PLFA profiles were examined using principal component analysis (PCA)

and the significance of results were assessed using a *post-hoc* analysis of variance (ANOVA) using STATISTICA version 9 (Statsoft, 2009).

**Table 6-1:** Basic properties of the three soils used.

| | Loamy Sand | Clay | Sandy loam |
|---|---|---|---|
| Grid reference | 26˚25'W, 52˚26'N | 25˚56'W, 52˚12'N | 26˚27'W, 52˚33'N |
| WRB[1] ref group | Cambisol | Stagnosal | Luvisol |
| Series name | Bearsted | Lawford | Maplestead |
| Sand % | 71.9 | 19.4 | 63.7 |
| Silt % | 16.4 | 24.2 | 18.9 |
| Clay % | 11.7 | 56.1 | 17.4 |
| Total organic matter % | 3.95 | 7.63 | 5.94 |
| Total organic carbon | 1.60 | 2.69 | 2.39 |
| Total carbon | 1.71 | 2.78 | 2.36 |
| Total nitrogen % | 0.16 | 0.295 | 0.21 |
| Density g $cm^{-3}$ | 1.25 | 1.19 | 1.09 |
| pH | 7.5 | 7.1 | 6.9 |
| Water content % | 0.98 | 10.2 | 1.31 |
| Water holding capacity mL $g^{-1}$ | 43.4 | 74.5 | 58.0 |

[1]WRB = World Reference Base.

## 6.4 Results and discussion

It was observed that all solvents and aqueous solutions were able to remove triclosan effectively such that no carry over was observed in any of the PLFA chromatographs. However some of the solvents changed the apparent PLFA profile of the soil. PCA projection for PLFA profiles from treatments and controls in the three soils are shown in Figure 6- 2. If the PLFA profiles are absolutely congruent

between any compared samples, they will occupy the same location in such an ordination. For the loamy sand soil (Fig 6-2a), and the sandy loam soil (Fig 6-2c), this was the case for those treated with a hexane or a 50% hexane/water mix, i.e. both these treatments resulted in PLFA profiles which were not statistically different from the control with respect to either PC1 or PC2 (Fig 6-2a,c) ($p>0.05$). The PLFA profiles derived from all other treatments were significantly different to the control ($p<0.01$), with the exception of the 50% MeOH: water mix which was not significantly different to the control in the sandy loam soil (Fig 6-2e). Rinsing with DCM and MeOH had the most significant effect on the extracted FAME chromatograms, which often showed depressed or missing peaks, relative to those from the respective control. These solvents, which are more polar than hexane, may cause lysis of microbial cells, releasing PLFAs prior to the PLFA extraction process. The PLFA profiles derived from the clay soil after solvent washing were most similar to those derived from the respective control (Fig 6-2e). Treatments with hexane, MeOH and all three aqueous solutions generated FAME chromatograms which were not significantly different to those extracted from control soils ($p>0.05$). The principal components derived from PLFA profiles extracted from soils treated with DCM or a 50% DCM/water mix were clearly separated from all other treatments and the controls, again confirming its relatively poor performance. There were no dominant loadings for individual PLFAs for any of the soils or treatments (Figs. 6-2b, d, f), showing that the discrimination between samples was not attributable to any particular lipid. These data then suggest that of the solvents tested, hexane was superior.

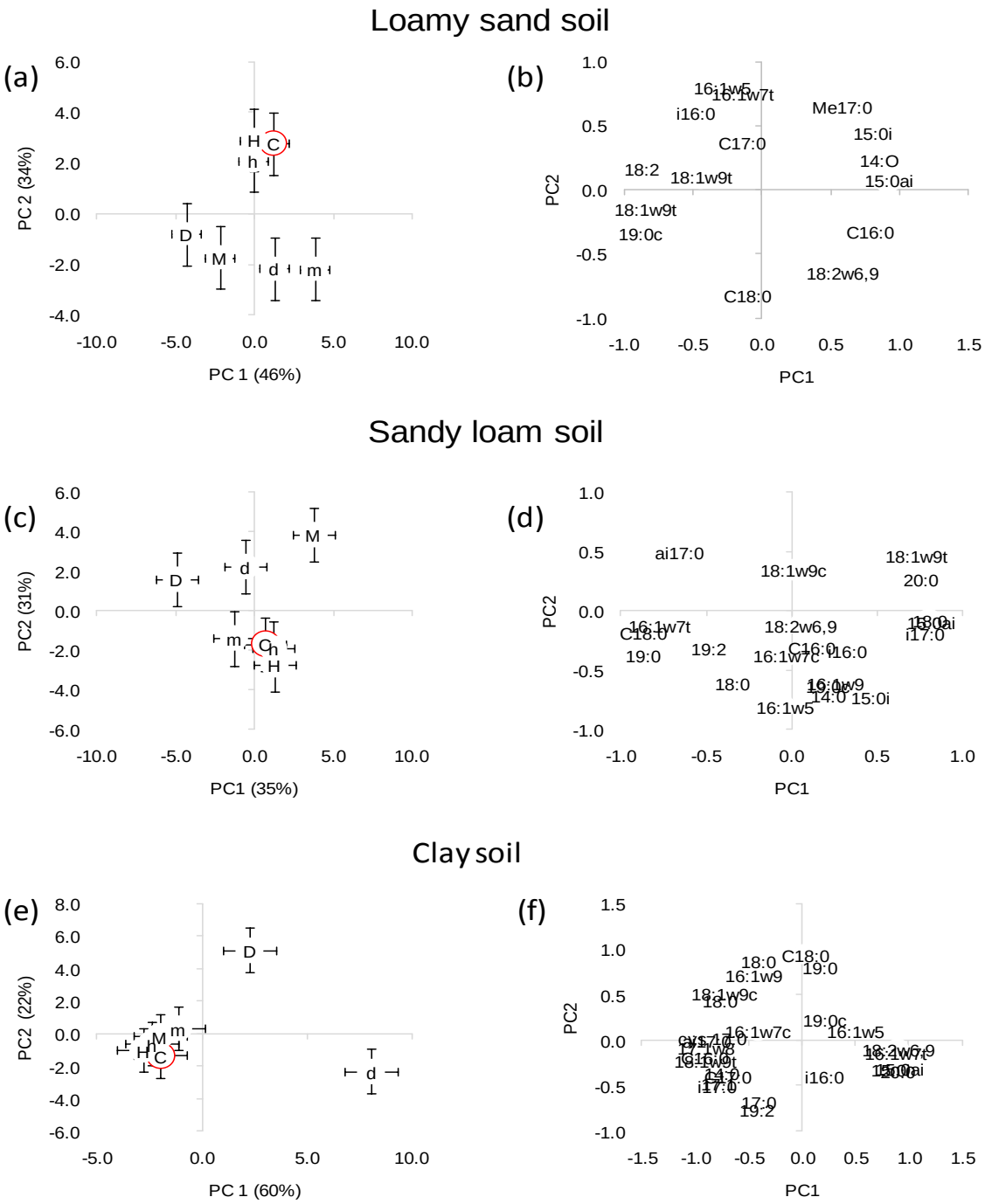


**Figure 6-2: PCA projection of the 1st and 2nd principal component plots derived from PLFA profiles in (a) loamy sand (c) sandy loam and (e) clay soils, washed with various solvents. C = no washing, H = Hexane, D =Dichloromethane, M =methanol, h = 1:1 mix hexane and water, d = 1:1 mix dichloromethane and water, m = 1:1 mix methanol and water. Plots b, d and f display the loadings for the differences visualised on PC1 and PC2. Error bars represent the standard error of the mean.**

A more stringent test is then to apply PCA to the profiles only derived from this solvent versus the control. In this case, statistically significant differences were apparent between the control and both hexane and hexane:water mix for all soils (Fig. 6-3a, c, e). However, this is inevitable given the nature of the PC technique which seeks to absolutely maximise differences between samples, and in these circumstances will be extremely sensitive to subtle differences in the chromatograms. That the three profiles ordinate in an equidistant manner for all three soils suggests that there is no inherent bias in the extraction involving hexane – if there were strong asymmetry in such an ordination, this would compromise the technique. Furthermore, the loadings are also widely distributed (i.e. no single PLFA dominates; Fig 6-3b, d, f) which demonstrates no systematic bias.

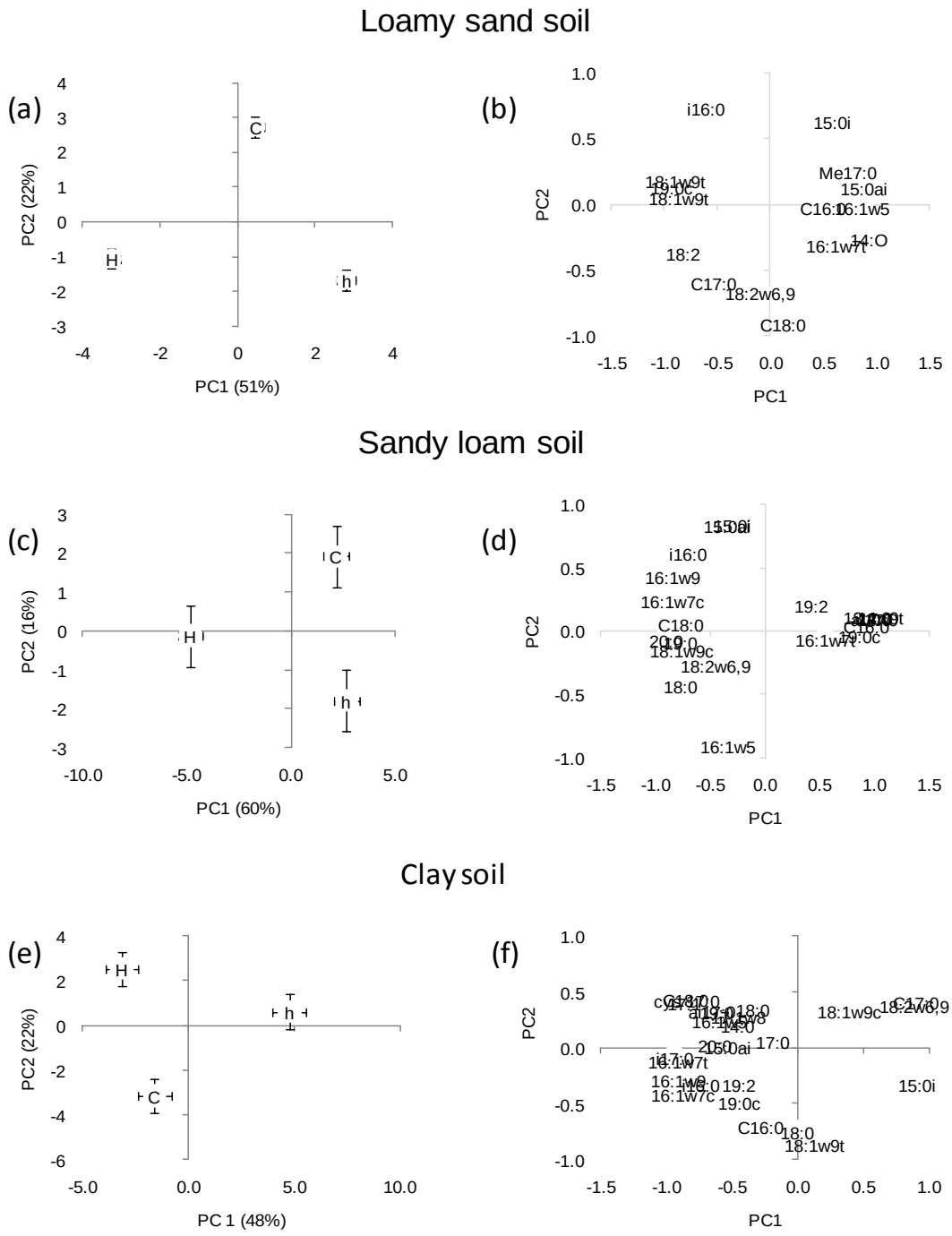


**Figure 6-3: PCA projection of the first and second principal component plots derived from PLFA profiles in (a) the loamy sand soil (c) the sandy loam soil and (e) the clay soil, washed with: H = Hexane, h = 1:1 mix hexane and water or C = no washing. Points represent means (*n*=3). Plots b, d and f display loadings for the differences visualised on PC1 and PC2. Percentage variation accounted for by principal component is shown in parenthesis on each axis. Error bars represent the standard error of the mean.**

Example phospholipid chromatographs from hexane-washed soils, when overlain with a chromatogram from an un-dosed, unwashed control (Fig. 6-4), demonstrate the high degree of fidelity between these two circumstances. Although miscible organic solvents such as methanol offer a straightforward way to remove organic contaminants from soil (Kawabata and Nakagawa, 1991), such solvents can be cytotoxic at high concentrations (Hugo, 1971; Salter and Kell, 1995) and affect PLFA profiles due to premature cell lysis making the fatty acids degrade prior to extraction. Those which are less damaging to cell membranes are usually more polar with a restricted solvating power which may make them less useful for removing hydrophobic contaminants. Another potential problem is the fact that different cell types or individual strains may vary considerably in their response to a solvent, even under the same conditions (Salter and Kell, 1995). Solvents can also alter the composition and chemistry of the soil by, for example, increasing contaminant solubility or altering cation exchange capacity (Mulligan *et al.*, 2001). The most promising results were obtained using hexane, which was able to adequately remove triclosan and did not significantly alter the relative abundance of microbially-derived fatty acids. This performance may be due, in part, to the fact that hexane is non-polar and forms reversed micelles in soil (lipids move in the presence of non-polar solvents so that the polar tails are protected in the centre of a water emulsion whilst the hydrophobic head will face out into the solvent), which may proffer some degree of protection for any phospholipids released (Cheng and Sabatini, 2001).

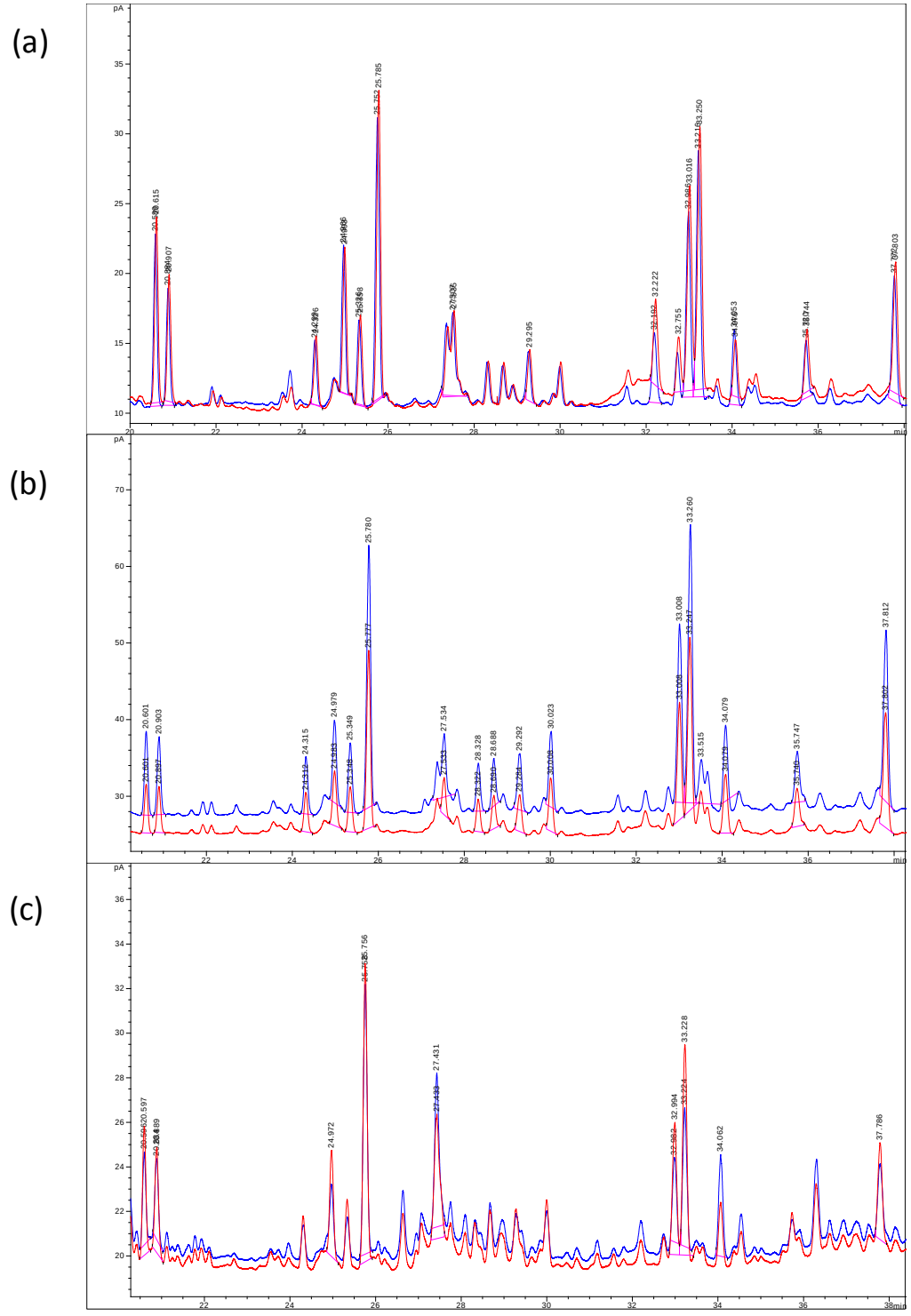


**Figure 6-4: Overlay of PLFA profile chromatographs from the loamy sand soil. Blue = Control chromatograph (No washing) and (a) Red = Soil washed with hexane: water mix (1:1), (b) Red = Soil washed with hexane: water mix (1:1) and (c) Red = Soil washed with hexane: water mix (1:1).**

## 6.5 Conclusions

This method describes a simple but efficient method for removing interfering lipophilic compounds to enable phospholipid fatty acid analysis without altering the soil phenotypic community. The method here was tested on triclosan only; however it has the potential for removing other hydrophobic chemicals from soil, thus enabling the detection of changes in microbial community composition using PLFA analysis under a variety of circumstances. However, further analysis would be required to confirm that this is the case with each contaminant of interest as different pre-treatment can result in diverse results. It is also important to ensure that all methodological conditions are maintained in each soil such as ensuring that soils dosed with toxins are washed in the same way as controls to avoid confounding factors.

# Chapter 7

## The effect of triclosan on microbial community structure in three soils

**Butler, E**., Whelan, M.J., Ritz, K., Sakrabani, R., van Egmond, R., 2011. The effects of triclosan microbial community structure in three soils. Accepted by *Chemosphere* subject to satisfactory revisions, March 2012.

## 7.1 Abstract

The application of sewage sludge to land can expose soils to a range of associated chemical toxicants. In this paper we explore the effects of the broad spectrum anti-microbial compound triclosan on the phenotypic composition of the microbial communities of three soils of contrasting texture using phospholipid fatty-acid (PLFA) analysis. Each soil type was dosed and subsequently re-dosed six weeks later with triclosan at five nominal concentrations in microcosms, including a zero-dose control. PLFA profiles were analysed using multivariate statistics focussing on changes in the soil phenotypic community structure. Additionally, ratios of fungal: bacterial PLFA indicators and cyclo: mono unsaturated PLFAs (a common stress indicator) were calculated. It was hypothesised that the addition of the bacteriocide triclosan would alter the entire community structure in each soil and in particular that the soils would show a higher fungal: bacterial ratio as we predicted that bacteria would be more vulnerable to triclosan than fungi after dosing. It was also hypothesised that the response to re-dosing would be, to a lesser degree, due to the onset of microbial acclimation to triclosan. The results suggest that the microbial community structure changed over the course of the experiment. However, the response was complex, with soil type and time as the most important explanatory factors. Principal component analysis was used to detect phenotypic responses to different doses of triclosan in each soil. There was a significant increase in the fungal: bacterial ratio with triclosan dose especially with concentrations of 500 and 1000 mg $kg^{-1}$ confirming the hypothesis that fungi are may be vulnerable to triclosan toxicity than bacteria. Futhermore, the PLFA response to re-dosing was negligible in all soils confirming that some acclimation of the soil microbial communities to triclosan addition had occurred.

## 7.2 Introduction

Soil microbial communities are essential in controlling nutrient cycling and soil organic matter decomposition (e.g. Harris, 2009; Wang *et al.,* 2008). The microbial community structure of agricultural soils can be influenced by several factors including soil texture (Bach *et al.,* 2010), moisture content (Williams and Rice, 2007), pH (Rousk *et al.,* 2010) and temperature (Norris *et al.,* 2002). In addition, microbial communities can be affected by agricultural management practices such as tillage (Helgason *et al.,* 2010) and can be exposed to a wide range of potential toxicants either directly through the application of pesticides, or indirectly through the addition of contaminated manure (Blackwell *et al*., 2009) or sewage sludge (Kannan *et al*., 2007; Heidler *et al*., 2006).

One terrestrial contaminant which has received recent attention in the literature is triclosan (e.g. Capdevielle *et al*., 2008; Reiss, 2009), an anti-microbial compound used in personal-care products. Triclosan is frequently detected in sewage-sludge as a result of partitioning during the waste-water treatment process. Aquatic studies have shown that triclosan can have a detrimental effect on algae at relatively low concentrations (Orvos *et al.,* 2002; Wilson *et al.,* 2003), and inhibited bacterial metabolism has also been observed (DeLorenzo *et al.,* 2008). In soils, triclosan has been shown to increase dehydrogenase activity (Ying *et al.,* 2007), and affect microbial respiration rates (Waller and Kookana, 2009; Butler *et al.,* 2011a). Triclosan concentrations as low as 4 mg $kg^{-1}$ have been observed to affect microbial populations and inhibit the degradation of other xenobiotic compounds (Svenningsen *et al.,* 2011). Increased persistence in soil may lead to an increased risk of leaching,

although the leaching risk is mitigated by a high affinity for soil solids (Gustafson *et al*., 1995). In a previous study (Butler *et al*., 2011a), we showed that very high doses of triclosan relative to environmental concentrations, altered soil functional behaviour by way of short-term respiration inhibition and depression of the microbial biomass. Soil response to subsequent re-dosing suggested microbial acclimation, with evidence that triclosan was being utilised as a substrate.

PLFAs are present in the membranes of all living cells but are rapidly decomposed by hydrolytic cellular enzymes after cell death. This means that the primary source of PLFAs in soil is living microbial cells. Extracted PLFAs can provide a phenotypic description of the associated soil community, reflecting species composition and abundance (Findlay, 2004). This method has been used extensively to track both spatial (Mele and Crowley, 2008; Ritz *et al.,* 2004) and temporal (e.g. Moore-Kucera and Dick, 2008; Hamel *et al.,* 2006) changes in community structure, and has also been used to determine the effects of pollutants on soil communities, including metals (e.g. Farrell *et al.,* 2010*;* Sverdrup *et al*., 2006; Tischer *et al*., 2008) and organic toxicants (e.g. Elsgaard *et al*., 2001; Bartling *et al*., 2009; Zhang *et al*., 2010).

Changes in community structure can potentially affect the associated function of the soil ecosystem. For example, fungi can secrete compounds capable of binding soil particles into aggregates which provide physical protection for soil organic matter (Van Groenigen *et al.,* 2007) and, thus, enhance carbon sequestration (Six *et al.,*

2006). Fungi and bacteria have different C/N ratios (Bååth and Anderson, 2003), which is important in controlling the magnitude and direction of nitrogen mineralisation or immobilisation during litter decomposition. There are also important differences between these microbial groups in terms of their observed reaction to different stressors, such as metals (Rajapaksha *et al.,* 2004), substrate starvation (Demoling *et al.,* 2008) and soil pH (Rousk *et al.,* 2010).

In this paper, we augment the results reported by Butler *et al*. (2011a) by evaluating the effect of triclosan addition on the composition of soil microbial community structure using phospholipid fatty acid (PLFA) profiling. We hypothesised that triclosan will have a different toxic effects on different components of the microbial community, resulting in an altered phenotypic structure after dosing. However, we also hypothesised that the microbial community will build up some resistance and resilience to triclosan dosing resulting in a less significant phenotypic response, in line with the respiration-inhibition response to re-dosing reported by Butler *et al*. (2011a). Although triclosan is a broad spectrum antimicrobial compound, it is likely to have a more potent effect on bacteria than on fungi, resulting in a relative increase in fungi as a result of dosing with high triclosan concentrations.

## 7.3 Methods

### 7.3.1 Soil microcosm preparation and sampling

A 70-day incubation experiment was conducted in soil microcosms. Methods employed in soil selection, soil sampling and soil preparation, microcosm set-up and

dosing are described in full by Butler *et al*. (2011a). Briefly, soil samples were collected from the upper 20 cm of three different agricultural soils (a sandy loam, clay, and loamy sand) at Silsoe Farm in Bedfordshire, United Kingdom. All sites have been under an arable rotation dominated by winter wheat and peas for over 20 years. Samples were refrigerated at 5°C immediately after collection, sieved moist to <2 mm, manually mixed, and air dried following standard methods (BS 7755 section 2.6: 1994 and BS ISO 11464 2006). Aliquots of each dry soil (50 g) were placed into 200 mL glass bottles and rehydrated to 60% maximum water holding capacity. Soils were left to adjust to the incubation temperature (20°C) for two weeks before being spiked with triclosan in 1 mL ethanol at nominal concentrations of 0, 10, 100, 500 and 1000 mg $kg^{-1}$ dry soil on Day 0 of the experiment.

Samples were taken from each microcosm daily for seven days and then on Day 14 for measuring respiration response (Butler *et al.,* 2011a). Soils were then left for six weeks before re-dosing with the same nominal triclosan concentrations and sampled as before for the first seven days and, again, on Day 14 after re-dosing. On Days zero, 2, 14, 56 and 70 (Day 56 is the day of re-dosing) additional 10 g soil aliquots were collected for PLFA analysis (PLFA extraction was undertaken after re-dosing on Day 56). These samples were placed into 50 mL glass vials, frozen at -80˚C for 48 hours, freeze-dried and stored at -20˚C until extraction.

### 7.3.2 Soil washing

Initial trials showed that residual triclosan can be extracted with PLFAs which then co-elutes with the fatty acids on the chromatogram at high concentrations, masking the PLFA peaks. A simple method of soil washing was, therefore, developed to remove the triclosan without affecting the phenotypic profile (Butler *et al.,* 2011b). Briefly, soil was shaken with 10 mL of hexane and 10 mL deionised water for two hours at 300 rpm followed by centrifugation at 2000 rpm for 5 minutes to remove the aqueous phase and dried overnight at 37˚C, frozen at -80˚C for a minimum of 24 hours and freeze-dried.

### 7.3.3 PLFA extraction

PLFA analysis was conducted following the method of Frostegård *et al.* (1993). In brief, phospholipids were extracted from soil using chlororform, methanol and a citrate buffer in a ratio of 1: 2: 0.8 (v: v: v) (Bligh & Dyer, 1959). Fatty acids were then fractionated from the other extracted lipids using normal phase solid phase extraction (SPE) and then derivatised by mild alkaline methanolysis to form fatty acid methyl esters (FAMEs) which were separated and detected using gas chromatography coupled with a flame ionization detector (GC-FID). An Agilent 6890N GC was used fitted with a split/splitless injector and an HP-5 (Agilent Technologies) capillary column (30m length, 0.32 mm ID, 0.25 µm film, and containing 5% phenylmethyl siloxane). Helium was used as the carrier gas (1 mL per min) and the FAMEs were separated using the following temperature program: 50°C for 1 minute (splitless hold time), increasing at a rate of 25°C per minute up to 160°C followed by a 2°C per minute rise to 240°C and a 25°C per minute increase to

310°C. Samples were injected (1 µL) using an auto-sampler with an injector temperature of 310°C. FAMEs were detected using FID operating at 320°C, and compared with known retention times to identify individual PLFAs using a Supelco 26 PLFA peak standard (Sigma Aldrich, Dorset, UK).

The fatty acid nomenclature used was that described by Frostegård *et al* (1993). The fatty acids *i*15:0, *a*15:0, 15:0, *i*16:0, 17:0, cy17:0, 18:1*ω*7 and cy19:0 were chosen to represent bacterial PLFAs (Federle, 1986; Frostegård *et al.,* 1993). The unsaturated PLFA 18:2*ω*6,9 was used as an indicator of fungal biomass (Federle, 1986; Zogg *et al*., 1997). A fungal: bacterial PLFA ratio was used as an indicator of changes in the relative abundance of these two microbial groups (Bardgett *et al*., 1996; Strickland and Rousk, 2010). This was calculated by dividing the mean RA for the fungal biomarker by the sum of the mean RAs of the bacterial PLFA indicators listed above. The ratio between the cyclo and mono-unsaturated PLFAs cy17:0/16:1ω7c and cy19:0/18:1ω7c was used as a stress indicator (Frostegård *et al.,* 2010).

### 7.3.4 Statistical analysis

Repeated measures analysis of variance and a *post hoc* fisher test was applied to the results of both the fungal to bacterial ratio and the stress indicator ratios to test statistical significance.

Each PLFA peak area was used to calculate total abundance in the complete PLFA profile for each sample. Peak areas for individual fatty acids were divided by the

total abundance to give a relative abundance (RA). For each soil type, in each treatment, at each time point, a mean RA was calculated from triplicate RA values. In order to facilitate data analysis using multi-variate statistics (i.e. to normalise the frequency distributions) mean RA values were then square root transformed to give root relative abundance (RRA). RRA values were analysed using a combination of principal component analysis (PCA) and repeated measures analysis of variance (ANOVA) using STATISTICA version 10 (Statsoft, 2010).

## 7.4 Results

### 7.4.1 Full factorial analysis

As a starting point, PCA was performed on all soils at all sampling times and at all nominal triclosan concentrations. However, third order interactions were observed in this analysis, making interpretation difficult. In particular, soil type made a major contribution to observed variability and masked other more subtle variations such as the effect of time and triclosan dose (See ANOVA Table 1). Time after dosing explained the next greatest proportion of the variance with triclosan dose also making a smaller but still highly significant ($p$>0.01) contribution. In order to simplify interpretation, consequently each soil was then considered separately with independent PCA and repeated measures ANOVA duly applied. In all cases there were significant time x treatment interactions (p<0.001).

**Table 7-1:** Repeated measures analysis of variance (ANOVA) comparing principal components 1 and 2 derived from the principal component analysis looking for variations in microbial communities due to the effects of soil type, triclosan concentration and time after dosing.

| PC1 | df | EMS | F | p |
|---|---|---|---|---|
| Soil | 2 | 39.3 | 44.9 | 5.55E-16 |
| [Triclosan] | 4 | 4.4 | 5.0 | 8.40E-04 |
| Time | 4 | 36.1 | 41.2 | 4.92E-12 |
| Soil* [Triclosan] | 8 | 4.1 | 4.6 | 4.39E-05 |
| Soil* Time | 8 | 22.3 | 25.5 | 0.00E+00 |
| [Triclosan]* Time | 16 | 5.5 | 6.3 | 1.49E-10 |
| Soil* [Triclosan] *Time | 32 | 3.8 | 4.4 | 3.25E-10 |
| PC2 | | | | |
| Soil | 2 | 27.8 | 59.1 | 0.00E+00 |
| [Triclosan] | 4 | 3.6 | 7.6 | 1.40E-05 |
| Time | 4 | 82.4 | 175.3 | 0.00E+00 |
| Soil* [Triclosan] | 8 | 1.4 | 3.1 | 3.21E-03 |
| Soil* Time | 8 | 10.2 | 21.6 | 0.00E+00 |
| [Triclosan]* Time | 16 | 4.1 | 8.8 | 6.66E-15 |
| Soil* [Triclosan] *Time | 32 | 2.6 | 5.5 | 2.20E-13 |

**df signifies the degrees of freedom (n-1), EMS is the error mean squared, F is the statistical F test result and p is the statistical p value of significance. p values marked with an* refer to a significant result with an alpha value of 0.01.**

### 7.4.2 Analysis based on soil type

The PCA plot for the loamy sand soil (Fig 1a) shows that time after dosing is clearly separated by PC1. The principal components (PCs) for all treatments on Day 2 were projected to the left of the plot and there was a progression rightwards in the ordination with increasing time to Day 56. PCs on Day 70 were, however, indistinguishable from those on Day 56. The effect of time in the sandy loam soil

(Fig 7-1c) was more complex. PCs derived from Day 2 samples (in all treatments, including the control) were clearly distinguished from those from Day zero, separated once again on PC1. However, on Days 14 and 56 the projections moved progressively back towards those apparent on Day zero. As in the loamy sand soil, the projections from samples collected on Days 56 and 70 were not separated by the PCA. The projections derived for the clay soil (Fig 7-1e) were also complex, in terms of the effect of time on the phenotypic community. Once again, profiles derived from all samples collected from all treatments on Day 2 were separated from those for Day zero on PC1. On Day 14 profiles for different treatments appeared to diverge and there is a general tendency to return to towards the projections for Day 0. However, the points for Day 56 appeared to be closer to those for Day 2 and, as in the other soils, there was no temporal separation between Days 56 and 70.

Although the dose of triclosan had a lower statistical explanatory power for the microbial community structure than soil type and time (Table 6-1), highly significant effects of treatment were still observed. In the loamy sand soil (Fig 6-1a) a treatment effect was apparent in the separation on PC2 of points derived from different triclosan doses on Day 14. Points derived from samples collected on Days 56 and 70 appeared to be separated by triclosan dose on PC1. All of the points from different treatments (including the control samples which received no triclosan) were grouped together on Days 0 and 2 (i.e. there appeared to be no treatment effect on these days).

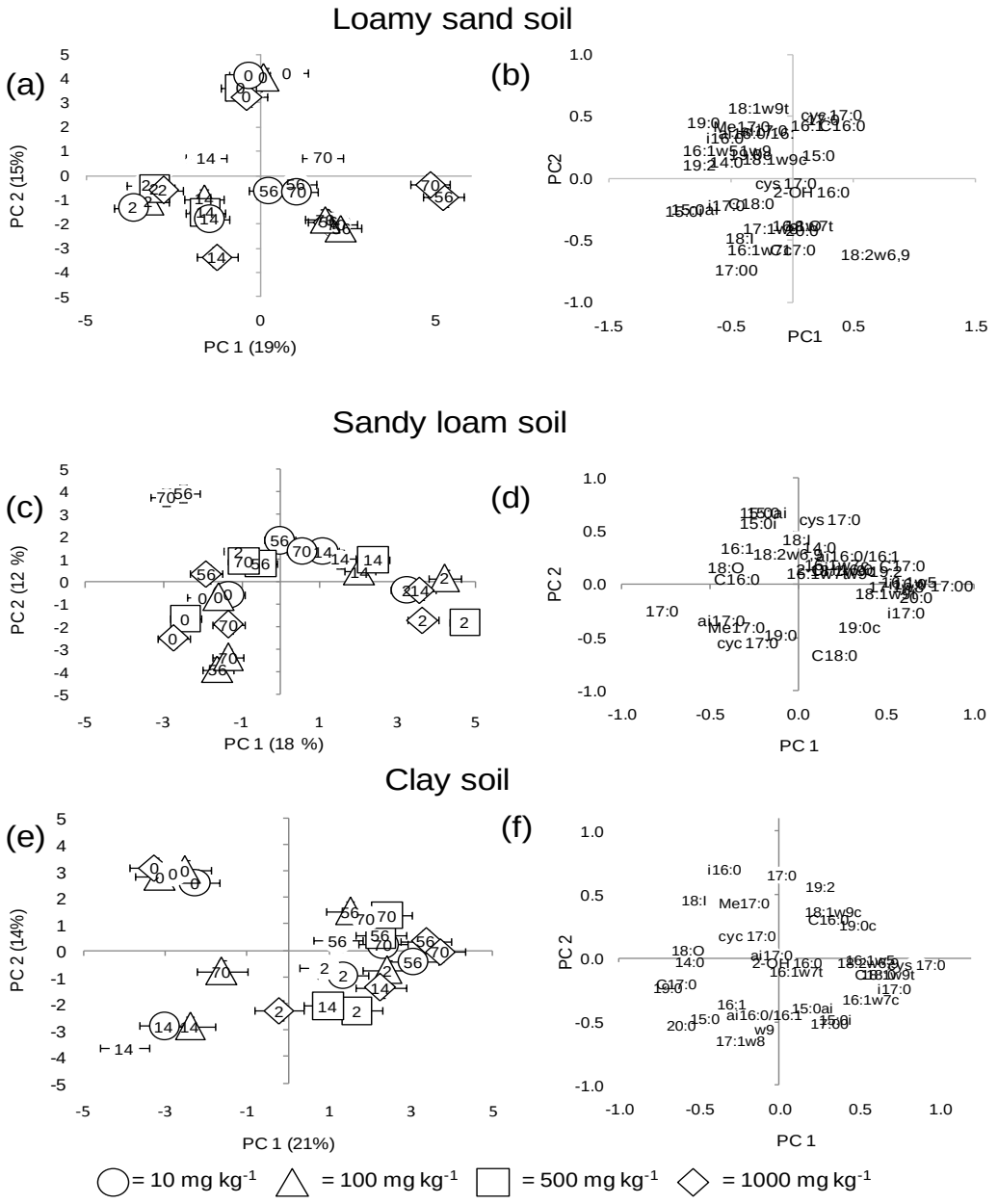


**Figure 7-1:** PCA projections of the first and second principal components (PC) derived from the PLFA profiles in the three soil types dosed with nominal concentrations of triclosan (No shape = control, ○= 10, Δ = 10□ = 500 and ◊= 1000 mg $kg^{-1}$). The number at the centre of the shape represents day after dosing (Days zero, 2, 14, 56 and 70). Error bars signify the standard error of the mean derived from repeated measures analysis of variance (ANOVA) on each principal component. Loading plots accounting for the variation seen on PC1 and PC2 for the b) loamy sand; d) sandy loam and f) clay.

A different treatment effect was observed in the sandy loam soil (Fig 7-1c). There was some separation of points on Day 0, with samples receiving 500 and 1000 mg $kg^{-1}$ triclosan doses separated on PC2. The projections for all treatments on Day 2 showed a significant separation from those on Day 0 and from one another.

It is interesting to note that the point derived for the control samples on Day 2 was still located relatively close to the Day 0 projections suggesting that a triclosan treatment effect may be responsible for this shift. A triclosan dose effect was also apparent on Day 14 with samples being separated on PC1 in order of increasing triclosan concentration. However, by Days 56 and 70 such treatment effects were not apparent. In the clay soil (Fig 7-1e) there was also strong evidence of a treatment effect. Day 2 projections were, once again, clearly separated from those for Day zero on PC1 and PC2 and there was separation of treatments on PC1. The dose-response effect was probably clearest on Day 14 where there was a separation of treatments on PC1 in order of increasing triclosan dose. As with the other soils, no treatment effects were evident after Day 56.

PLFA loadings serve to identify the extent to which the variability observed can be explained by specific lipids. There were no dominant loadings for individual PLFAs for any of the soils or treatments which suggest that the discrimination between samples was not attributable to any particular fatty acid.

### 7.4.3 Fungal to bacterial marker ratio

The fungal to bacterial marker ratio (FBR) describes the relative abundance of fungi and bacteria in the phenotypic community. In the loamy sand soil (Fig 7-2a) there was an increase the FBR in all treatments on Day 2, including the control. There were no further significant changes in the ratio in any of the treatments until after re-dosing on Day 56 and Day 70, when there was a significant ($p$<0.01) increase in the ratio for treatments receiving 100 mg triclosan $kg^{-1}$ and above. In the sandy loam soil (Fig 3b) the FBR in the control was higher than in the loamy sand and did not increase significantly on Day 2, suggesting a reduced ethanol effect in this soil type. By Day 14, and on subsequent days, there was a clear increase in FBR with triclosan dose. The maximum ratio observed was approximately 0.3. Note that the only statistically significant increase in FBR (repeated measures ANOVA) occurred in the 1000 mg $kg^{-1}$ treatment on Days 14, 56 and 70 and in the 100 and 500 mg $kg^{-1}$ treatments on Days 56 and 70 only. In the clay soil (Figure 7-3c), the FBR was initially similar to that observed in the sandy loam soil and increased after Day 14 in the 500 and 1000 mg $kg^{-1}$ treatments. The peak FBR (0.7) was greater than that observed in the sandy loam soil but less than that measured in the loamy sand. The repeated measures ANOVA showed that the 1000 mg $kg^{-1}$ treatment had a significant effect from Day 2 onwards. There was a significant treatment effect on the FBR at all doses on Day 14 but by Days 56 and 70, the FBR was significantly elevated only in the 500 and 1000 mg $kg^{-1}$ treatments.

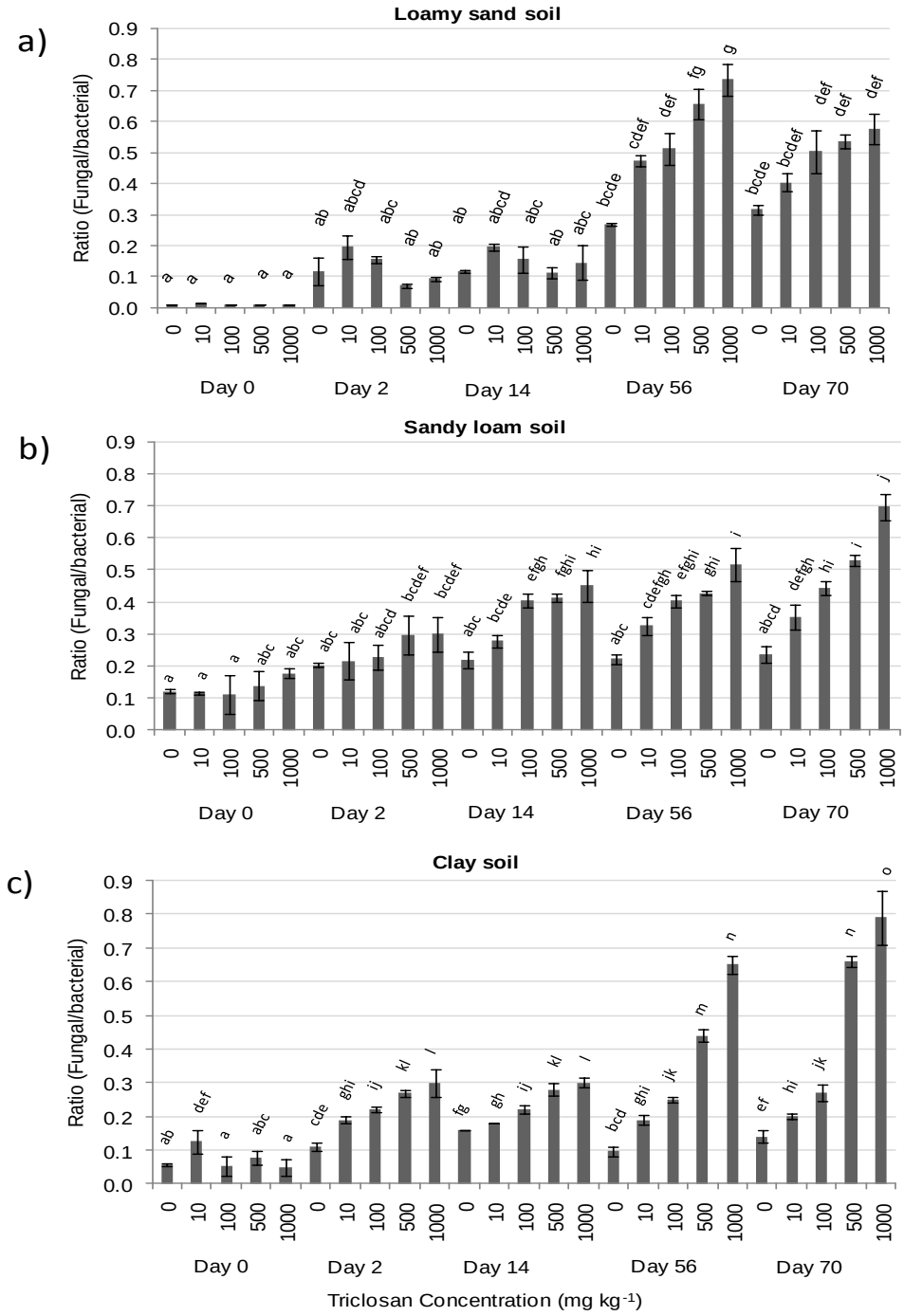


**Figure 7-2 The ratio of fungal to bacteria PLFA markers (FBR) in each soil: a) loamy sand; b) sandy loam and c) clay, at all nominal concentrations of triclosan on five sampling occasions. Error bars signify the standard error of the mean. Repeated measures Analysis of variance (ANOVA) with Fisher's Least Squares (LSD) test produced letters a-j, showing significant between soil and between triclosan concentration treatment differences at $p < 0.01$.**

### 7.4.4 Stress indicators

For the CMR in the loamy sand soil (Figure 3a), a repeated measures ANOVA showed that time after dosing was the most statistically significant factor controlling this ratio ($p$<0.01). The effect of triclosan dose on CMR in this soil was not statistically significant ($p$>0.05), although visually, treatments receiving higher nominal triclosan did tend to have higher CMR values. The ANOVA for the sandy loam soil (Figure 3b) showed that both time of sampling and triclosan dose had statistically significant ($p$<0.01) effects on CMR. Although the triclosan concentration effects are clear at high concentrations (Figure 3b), below 100 mg $kg^{-1}$ there is no notable triclosan effect. The initial ratios are higher in this soil than in the loamy sand soil (including the control) suggesting that the addition of ethanol and/or other pre-treatment steps may have elevated CMR levels in this soil. In the clay soil, CMR is also significantly affected ($p$<0.01) by both sampling time and triclosan dose. This soil shows the clearest concentration effect of all three soils (Figure 3c), although CMR levels tended to be lower in this soil than in the other two, except at the highest (1000 mg $kg^{-1}$) triclosan concentration.

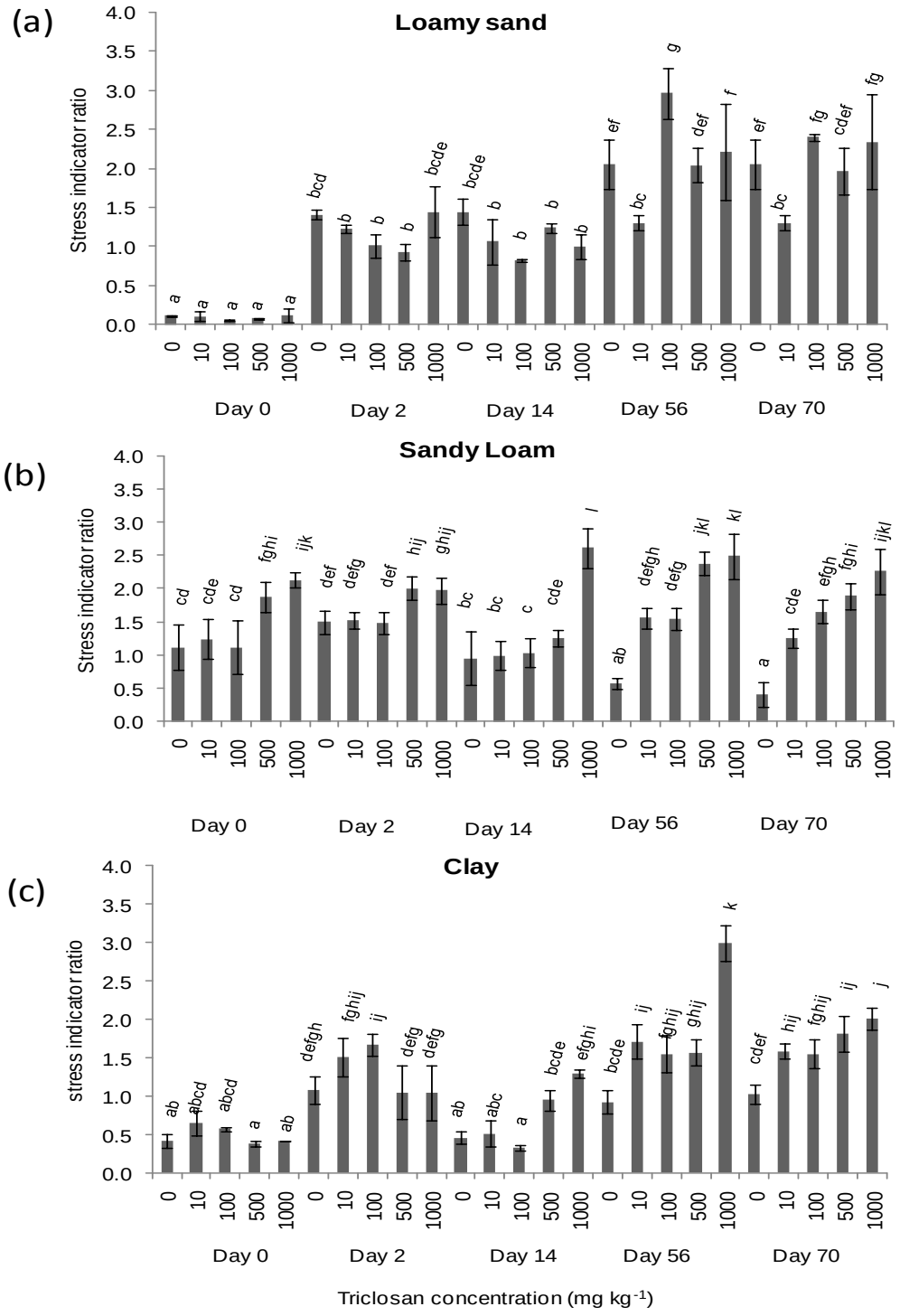


**Figure 7-3: The ratio of cyclo: mono unsaturated PLFA markers (CMR) in each soil: a) loamy sand; b) sandy loam and c) clay, at all nominal concentrations of triclosan on five sampling occasions. Error bars signify the standard error of the mean. Repeated measures Analysis of variance (ANOVA) with Fisher's Least Squares (LSD) test produced letters a-j, showing significant between soil and between triclosan concentration treatment differences at $p < 0.01$.**

## 7.5 Discussion

Several factors may be responsible for altering the soil phenotypic community structure as observed here. The most significant factor in this study was soil type. This would be driven by various factors including pH (Bååth and Anderson, 2003) and organic carbon content (Patra *et al*., 2008) which are shown to have strong effects on microbial community structure. The influence of soil type was so great that it masked the more subtle effects caused by the addition of triclosan. When data sets for each soil type were analysed separately, PCA was able to identify significant changes in the microbial community structure as a result of experimental manipulation including adding triclosan. One of the clearest observations that can be made is that the microbial community changed over time, independent of triclosan addition, with control samples which received no triclosan also separated by time (Figure 1). Actions such as stirring and sieving the soil can result in physical disturbance to the soil system which can result in stress responses from the microbial community (Dannenberg *et al.*, 1997). Furthermore, incubating at 20ºC, as we did here, can also alter the microbial biomass and its composition (Joergensen et al., 1990), resulting in a change in the phenotypic structure. Finally, the addition of ethanol as a carrier solvent to introduce triclosan to the microcosms initially may also have acted as a stressor and or as a substrate, shifting the microbial community structure initially (Griebel and Owens, 1972). The PCA captured the effect of time after dosing in all three soils (Figure 1). The samples taken at a particular time were often grouped together on the PCA, with the notable exception of those collected after Day 56, suggesting that sampling time was less important after re-dosing. This could be because the soil microbial community may have already been on a common trajectory in response to the severe disturbance induced in preparing the

microcosms and may have become settled by this time. The PCA for the sandy loam soil and the clay soil (Figure 7-1, panels c and e), did not clearly distinguish some of the Day 14 samples from those taken at a later date suggesting that, in this soil, the effects appear to subside quicker than in the loamy sand soil.

The effect of triclosan addition on the phenotypic community is also evident in the PCA plots (Figure 7-1), although not on all days in all soils. Cases where there is a clear separation of points by treatment with separation distance from the control increasing with increasing nominal dose, such as on Day 14 in the sandy loam and clay soils (Figure 7-1, panels a and e), suggest that phenotypic shifts have resulted from triclosan dosing. It should be noted that the time of peak respiration inhibition reported by Butler *et al*. (2011a), in the same experiment as the one we describe in this paper, was generally between two and four days after triclosan addition. It is unsurprising that some changes in the composition of the microbial community should have accompanied this inhibition (along with the coincident depression in microbial biomass) given what we know about the cytotoxic nature of triclosan. The response of the PLFA profiles to triclosan addition in the sandy loam soil was very different. A dosing affect was apparent on Day zero, suggesting that this soil may be more sensitive to triclosan than the others. In addition, treatment effects were also observed Days 2 and 14, with relatively little displacement of the control projection indicating that the observed shifts are probably a result of triclosan addition than of ethanol addition. Interestingly, the projections for the highest dose treatments on Day 14 appear to be closer to their respective positions on Day 2 than those for lower dose treatments.

PLFA profiles in soils sampled after re-dosing (Days 56 and 70) were similar and it was not possible to identify clear dose or time effects (points on Day 56 are located in very close proximity to those obtained on Day 70). The respiration inhibition data from the same experiment reported by Butler *et al.* (2011a) showed a repressed inhibition response in all soils following re-dosing compared to the initial dosing, suggesting that all three soils had were more resistant to triclosan toxicity (i.e. that the microbial community had acclimated to the stressor).

One of the clearest and most explicable changes observed in the PLFA profiles obtained from the experiment was the FBR response (Figure 7-2), with increased triclosan dose corresponding, in general, to higher FBR values. This confirms an hypothesis that fungi should be more resistant to triclosan exposure than bacteria because it targets the F*abI* gene which controls bacterial fatty acid synthesis. Fungi use a different fatty acid synthesis pathway which is not controlled by this gene (Carr *et al.,* 2011). There are exceptions in bacterial systems which can go some way to explaining why not all bacteria will be equally affected. For example, there are some bacterial strains which can over-express the F*abI* gene, causing mild resistance to triclosan or express a different gene altogether (e.g. F*abK*) in species such as *Bacillus spp*. (Wilson *et al.,* 2008), or contain pumps able to remove triclosan from cells, such as in *Pseudomonas aeruginosa* (Chuanchuen *et al.,* 2003). As with the PCA data, the FBR data suggest that there was some effect of sample preparation and or treatment with ethanol since the controls also displayed an increase in FBR. Ethanol may be preferentially used as a substrate by fungi or it may be more toxic to

bacteria than to fungi, promoting a change in the FBR. It should be noted that increases in FBR are most prevalent at high nominal concentrations (including after re-dosing), implying that lower concentrations of triclosan (i.e. those which would be expected following sewage sludge application) have little, if any, effect on FBR.

Overall, the microbial phenotypic community results reported here are consistent with the observations of respiration inhibition and microbial biomass changes reported by Butler *et al*. (2011a). In the respiration inhibition data, the clay soil showed the most rapid and significant initial response to triclosan addition but most resistance (*sensu* Orwin and Wardle, 2004) to triclosan re-dosing. This was explained, in part, by a lower triclosan bioavailability resulting from a higher organic carbon content and larger fraction of smaller pore sizes in this soil. The fact that PLFA profiles in the loamy sand soil were responsive to triclosan dose also supports the respiration inhibition data. This soil has the lowest organic matter content of all three soils making hydrophobic organic pollutants potentially more bioavailable. The PCA shows that the microbial community in the sandy loam was affected on Days zero and 2. This is also evident in the extent of respiration inhibition observed on Day 2 (Butler *et al.,* 2011a) which was greater than in both the loamy sand and clay soils. However, the Day 2 effect is not evident in the FBR values, suggesting that this initial response in PLFA profile may not be due to a change in the relative abundance of fungi and bacteria. It also suggests that fungi and bacteria are affected to a similar extent by the initial exposure to triclosan. Furthermore, the PCA loadings (Figure 7-1, panels b, d and f) show that the fungal biomarker (18:2ω6,9) was not affected in this soil type suggesting that the main changes in both

respiration inhibition and in the phenotypic profile were due to effects on the entire microbial community as opposed to a single organism type. The FBR appeared to be most responsive in the sandy loam soil. This can be explained, in part, by the pH of this soil (6.6 compared with 7.1 in the clay soil and 7.5 in the sand soil). At increasing pH more triclosan will be present in its phenolate anion form, which is less toxic as well as being potentially more mobile in the soil than the neutral triclosan molecule (Kwon *et al.,* 2010).

Bacteria will change their cell membrane composition in response to stressful environmental conditions (Navarro-Llorens *et al.,* 2010). The relative concentration of particular PLFAs, such as those used to calculate the CMR can, therefore, be used as "stress indicators". The CMR levels in all the soils examined in this study suggest that more stress may have been induced from ethanol spiking than from triclosan dosing. A notable exception was the 1000 mg $kg^{-1}$ treatment in which elevated CMR levels were observed in all three soils. It is very hard to distinguish stress changes in the cell membrane from overall changes in community structure (Frostegard *et al.,* 2010). Therefore, although it appears that triclosan induced little effect on the CMR, it did result in a significant depression in microbial respiration and microbial biomass. The changes in the FBR also suggest that community structure was probably affected by increasing exposure to triclosan.

Although PLFA analysis provides useful insights into the composition of the microbial community in soil and its response to stressors, it has a number of limitations. Using

multivariate statistics such as PCA can only answer one specific question: has there been a change in the entire microbial community as a response to a specific treatment? Any other interpretations are susceptible to misinterpretation (Frostegård *et al*., 2011). Using specific biomarkers can be indicative of species changes. However, caution should be exercised since biomarkers in the literature do not always agree. For example, the PLFAs cy17:0 and cy19:0 are commonly used as indicators of the presence of Gram negative bacteria, although both of these biomarkers are found in abundance in some Gram positive bacteria (Schoug *et al.,* 2008). Similarly, stress indicators should also be employed with a certain degree of caution as the assumption that the change between cyclo and mono PLFAs is due to microbes altering the composition of their cell membranes in response to stress could, in fact, be due to changes in species composition (Frostegård *et al*., 2011). Molecular techniques such as nucleic acid extraction (DNA and RNA) and associated fingerprinting techniques all have the potential to provide more information about the abundance of different organisms and the roles that these organisms play in the execution of key functions. However, relatively little work has been done, thus far, on the response of soil microbes to toxic stress from anti-microbial compounds such as triclosan using these techniques.

## 7.6 Conclusions

The respiration study of Butler *et al*. (2011a), showed that the addition of triclosan to soils in the same experiment as that reported here produced a clear inhibition of both basal and substrate induced respiration, especially at high nominal concentrations. Respiration rates subsequently recovered (≥ control) after less than two weeks.

When the soils were re-dosed with triclosan, the extent and duration of respiration inhibition was much less than following initial exposure, suggesting microbial acclimation. Furthermore, subsequent elevation of respiration *cf* control suggested some utilisation of triclosan as a substrate. The PLFA analysis presented here from the same experiment provides additional insight into the drivers for the observed respiration inhibition by elucidating some of the changes in microbial community structure which occurred in parallel. PCA projections showed that there was a temporal shift in the microbial community structure of all three soils, which may have been the result of pre-dosing disturbance. The PLFA data support the observation of damped respiration inhibition to re-dosing, especially in the clay soil, supporting the interpretation of microbial acclimation to triclosan. Although there were clear treatment (dose) related differences in the PLFA profiles extracted from samples taken after the initial application of triclosan, re-dosing resulted in significant treatment effects in only a few (high dose) treatments. The effect of triclosan dosing on FBR and CMR levels was clearer than the overall (complex) changes to the PLFA profiles, although other factors, such as ethanol addition and soil physical properties (e.g. organic carbon content and pH) also appear to be important.

It should be noted that the doses of triclosan employed in this study are very high relative to expected exposures from the operational application of sewage sludge to land (Kannan *et al.,* 2007). The intention of our study was to evaluate the response of the soil system to perturbation rather than to evaluate the risks associated with triclosan exposure in the field. At the lowest dose used here, both respiration inhibition and PLFA effects were relatively minor which suggests that significant

changes in the composition and function of the microbial community will probably not be manifested at typical exposure levels (< 1 mg $kg^{-1}$).

# Chapter 8

## Integrated discussion

## 8.1 Integrated Discussion

Many hydrophobic pollutants can be emitted to agricultural soils if sewage sludge is used as a fertiliser. The fate and effects of pollutants in such receiving environments are relatively poorly understood compared with our knowledge of chemical behaviour and impact in surface waters. One chemical of particular concern is triclosan because it has antimicrobial properties which could affect important soil functions. Triclosan is hydrophobic, which means it will sorb appreciably to organic solids and is not readily biodegradable (i.e. it does not pass the OECD ready test (OECD 301), although it is inherently biodegradable. It is also used extensively in personal care products. These factors have prompted considerable attention in the literature with respect to its environmental profile. In recent years, this attention has shifted away from the water environment to terrestrial systems. In the last 5 years in excess of 70 papers have been published on the fate and effects of triclosan in soil. Nethertheless, a number of important knowledge gaps remain.

1. Most previous fate studies are laboratory based. These small scale incubation studies are generally good for identifying the effects of controlled variables. However significant differences can exist in chemical behaviour between laboratory and field. This has been the case in terms of calculating degradation half-lives and bound residue formation (Al-Rajab *et al*., 2009), and can possibly account for the wide range of reported degradation rates (between 18 days; Ying *et al*., 2007 and 107 days: Lozano *et al*., 2010). There are several potential reasons for this including the fact that soil temperature and moisture content in the field are highly variable (Waria *et al*., 2011) and often more limiting to degradation than the high temperatures and near optimal moisture

contents often maintained in laboratory studies. In addition, field soils amended with biosolids can contain anaerobic microsites (e.g. at the centre of aggregates) in an otherwise aerobic system (Al-Rajab *et al*., 2009). If triclosan diffuses into these microsites (which may also be physically protected from microbes) it can avoid degradation.

2. There have been very few attempts to look at triclosan metabolite formation in soil, especially methyl-triclosan, which has previously been identified in aquatic samples (Halden and Paull, 2005), fish (Balmer, 2004) and recently in soil (Waria *et al*., 2011).

3. There are no studies comparing the effects of soil type on either the fate or the effects of triclosan in the terrestrial environment. Various soil physical and chemical properties (i.e. soil texture, pH, organic matter content and moisture content) can influence the degradation and movement of compounds, in addition to any effects on soil microbial community structure and function.

4. Very few studies have examined the effects of triclosan on soil microbial functions. Some studies do exist, looking specifically at phosphatase activity (Liu *et al*., 2009), dehydrogenase activity (Waller and Kookana, 2009) and microbial respiration (Svennigsen *et al*., 2011). However these studies are acute short term studies and there is currently no information relating to long term studies or the importance microbial resistance and resilience to triclosan.

5. There are no studies looking at the effects of triclosan on the soil microbial community structure. The few effects studies which have been published (mentioned above) indicate that triclosan does alter soil microbial functions in

some way. However it is currently not understood whether triclosan, affects the entire microbial community or whether certain species can gain advantage in triclosan enriched soils whilst others are adversely affected.

6. No studies have looked at the effects of re-dosing the soils with triclosan in terms of soil microbial function or community structure. It is currently unclear whether the response to re-dosing will be the same as that observed on receiving the initial dose or whether the soil microbial community can adapt or acclimate to dosing.

It was the aim of this thesis to better understand the fate and effects of sludge-borne pollutants in soil with a specific emphasis on triclosan, and try to address the knowledge gaps listed above to contribute to the better understanding of the fate and effects of organic contaminants in the terrestrial environment.

In Chapter 2 some of the knowledge gaps with respect to the fate of triclosan in biosolid amended soils were addressed, specifically mineralisation, primary degradation and the formation of bound residues. This laboratory based incubation study used radio-labelled triclosan to trace the fate of triclosan in soils and biosolid amended soils over time. Triclosan was very slow to mineralise with less than 7% mineralisation observed after 6 months. When triclosan was added to the soil with biosolid, mineralisation was slower than when triclosan was directly applied to the soil. The fact that triclosan degradation was microbially mediated was confirmed by the absence of mineralisation in the sterile control. Triclosan was difficult to extract

from the soil after the 6 month experiment. Sequential solvent extractions resulted in less than 36% of the applied $^{14}C$ being extractable from the soil. This was attributed to triclosan forming bound (non-extractable) residues in the soil which were either spatially or temporally unavailable for extraction and presumably also biodegradation and the exertion of toxicity effects. The most important conclusion drawn from this experiment was the extent to which triclosan was converted into its metabolite methyl-triclosan. This was observed for the first time in soil using radio-HPLC. Between 50-70% of the extractable $^{14}C$ was found in the methylated form by the end of the experiment, equating to between 18-26% of the originally applied $^{14}C$ compared to between 7-15% which remained in parent triclosan form. Methyl-triclosan formation began as early as 14 days after application to the soil, suggesting relatively rapid primary degradation of triclosan in soils. This study also helped to understand how soil physical and chemical properties affect degradation and mineralisation of organic pollutants in soil. Mineralisation was initially slower in heavier soils (with higher clay and organic matter content), although when applied with biosolids the mineralisation pattern was very similar suggesting that mineralisation was controlled more by interactions with the sludge matrix in this case.

There were several areas where improvements could have been made. Most importantly the experiment lacked a positive control containing a radiolabelled substance such as glucose, which mineralises at a known rate and could have been used to validate the mineralisation rate observed in this study. Additionally although the water content of the soil was adjusted at the beginning of the experiment, there

was no way to ensure that satisfactory water content had been maintained throughout. The extent to which these factors altered the results is unknown. The soils were aerated daily by removing the lids and exposing the soils to the air. However, this method is not necessarily good at maintaining good aeration and there was no attempt to monitor aeration status. Furthermore, removal of lids exposed the sterile controls to non-sterile conditions. The experiment could be repeated to address these points and ensure that the results were conclusive. Several additional experiments could also be carried out, for example the degradation time study looking at the formation of bound residues over time along with primary degradation or transformation of triclosan. For a better all round picture, the work on metabolite formation could have been conducted in all three soil types to see processes differ in the different soils. Additionally, only one triclosan concentration was looked at in this study. It would help to see whether concentration affects mineralisation, degradation, transformation and the formation of bound residues. For completeness, it would have been advantageous to use spectroscopic techniques (e.g. mass spectroscopy) to be able to identify some of the other degradation metabolites observed in the radio-HPLC chromatographs.

The analytical method which was developed, described in Chapter 3 was sensitive, robust and repeatable. Very good removal of triclosan and methyl-triclosan was observed from soils and biosolids using the ASE method and excellent levels of detection and quantification were observed using GC-MS. It is possible that by using a derivatising agent, there would have been less sorption of the compounds to the GC equipment resulting in better levels of detection being achieved. Advances in

analytical chemistry have meant new forms of liquid-chromatography such as ultra performance liquid chromatography (UPLC) coupled with non MS detectors such as tunable ultraviolet detection are able to detect both triclosan and methyl-triclosan. This method has not been used to quantify these compounds in soil but it is still a promising development. In addition to improved detection methods, there are also alternative extraction techniques which passive samplers that extract and concentrate samples *in situ*, such as polydimethylsiloxane fibre samplers (PDMS). Other techniques have been developed that use a dispersive liquid to optimise a liquid-liquid extraction, which has had higher efficiencies than using SPE. These new developments mean that with additional effort, lower levels of quantification could be seen with a lower cost and less environmental impact from excessive solvent use. However, the method developed here was more than sensitive enough for the task to which it was put.

The lack of realistic field data is a major problem in the understanding of triclosan fate. The field experiment detailed in Chapter 4 attempts to bridge this data gap. Triclosan degradation rates in the field were observed to be seasonal with very slow degradation in the cold and wet winter months but rapid in the warmer summer months when the soil became warmer and better aerated. Other soil physical and chemical properties such as organic matter and clay content and soil pH also appeared to influence triclosan degradation. Degradation was coincident with the formation of the metabolite methyl-triclosan. This is the first time that methyl-triclosan formation has been observed in field scale experimentation where the only form of triclosan applied has been through the addition of sewage sludge. Although the

majority of the triclosan had degraded into its methylated form by the end of the study, a mass balance revealed that there was very little total loss of the triclosan applied after one year. The data confirm previous studies which suggest that methyl-triclosan is more persistent than triclosan. As well as degradation, triclosan was observed to move through the three different soil horizons studied. This movement appeared to be seasonal and controlled by climatic factors such as soil moisture content (which, in turn, controls the hydrological response of the soil to rainfall). This suggests that triclosan is susceptible to leaching despite being relatively hydrophobic. It may be that some leaching in particulate or colloid form. The hydrophobicity of methyl-triclosan means that it is significantly unlikely to leach in its dissolved form. Its appearance at lower depths is principally attributed to the degradation of leached triclosan although some movement may have occurred in colloidal form. In addition, some of the chemical movement in the soil could have been due to bioturbation as well as leaching. Note that although some triclosan moved through the soil, after 12 months there was still between 28 and 50% of the originally applied dose in the top 10 cm of soil.

There are several areas where the field experiment could have been improved. For example, it would have been useful to look for other metabolites in the soil (as identified in Chapter 2), in order to try to get the broadest possible picture of triclosan degradation in the terrestrial environment. Some of the observations were difficult to explain. For example, it is difficult to explain why triclosan concentrations in the 20-30 cm layer continued to increase even during the summer months when leaching would not be expected. This could have been due to bioturbation. Further work

would be needed to confirm this hypothesis. It is possible that numerical modelling of chemical behaviour could help to explain some of the observations. This would be facilitated by continuous monitoring of soil temperature and moisture content with depth in each plot. Although soil moisture content was determined gravimetrically in samples, this was too infrequent to validate effectively hydrologically and related to chemical fate modelling. It would have been beneficial to continue sampling over the following year to try to determine the longer term fate of triclosan and methyl-triclosan in the field. This could have been monitored in two ways:

- Continue the same sampling regime to determine if methyl-triclosan begins to degrade
- Apply an additional dose of sludge to the soil to determine if degradation or movement patterns differ due to microbial acclimation to the repeated triclosan exposure.

The remaining chapters focussed on the effects of triclosan on microbial function and community structure. Chapter 5 examined the effects of triclosan on respiration and microbial biomass. Triclosan appeared to inhibit both respiration and biomass such that the inhibition was proportional to the nominal dose applied. However, both respiration and biomass recovered to levels equal to or greater than that measured in the control soils suggesting that the soils were resilient to triclosan dosing. On re-dosing the soils with the same nominal concentrations of triclosan, the response observed differed to the original response. There was a less inhibition overall and, at low doses, triclosan itself appeared to be utilised as a substrate as evidenced by the stimulation of respiration at low doses. This suggests that some soil microbes were becoming resilient to triclosan which could be evidence of acclimation. There were

several parts of this study which could have been improved. Firstly, high concentrations of triclosan were used (1-1000 mg $kg^{-1}$) which significantly exceed the expected environmental concentrations in soils receiving sludge (between 1-50 mg $kg^{-1}$ in biosolids and 0.02-0.8 mg $kg^{-1}$ in soils after receiving biosolids). If repeated it would be beneficial to dose with concentrations at or around the predicted environmental concentrations as opposed to such high doses used. Recent studies have suggested that part of the toxicity of triclosan in the aquatic environment may be induced by methyl-triclosan. It would therefore have been useful to have done a simultaneous study using methyl-triclosan in place of triclosan so that results could be compared. Alternatively, by destructively sampling and analysing the sample using the method described in Chapter 3 (GC-MS). The relationship between concentration (of both triclosan and methyl-triclosan) and effect could have been better established rather than relying on nominal dose. There are many different ways in which the effects of a compound on soil function can be tested. For example certain enzymes such as dehydrogenase or phosphatase can be monitored. Although triclosan was observed to affect biomass and soil respiration, it is not known whether other soil functions were affected and whether these would recover or acclimate in the same way.

Chapters 6 and 7 helped to identify whether the recovery of respiration and biomass and the different responses to re-dosing could be explained by changes in microbial community structure. The analysis (phospholipid fatty acid: PLFA) in Chapter 7 could not have been undertaken without the development of the soil washing method in Chapter 6. It was observed that triclosan was eluted from the GC column at the same time as some of the fatty acid methyl-ester peaks but at greater concentration,

masking them and making the interpretation of the PLFA profiles impossible. A simple method of solvent washing the soil was developed and employed to remove the triclosan from the soil without affecting the soil phenotypic structure. The PLFA data (Chapter 7) tell a complex tale. A temporal shift in the soil community structure was observed, which may have been due to factors other than the addition of triclosan. Since the shifts were also observed in the control soils, the initial disturbance of the soil prior to the addition of triclosan could have been influential. Nethertheless, there was still a definite triclosan effect which was related to the dose administered, with the greatest dose having the greatest impact on the community structure. As well as a dose related response, there was evidence of post dosing recovery in some of the soils. The results of this study also confirmed the findings of the respiration inhibition study in that the second dose had a lesser effect on the soil community structure, confirming the hypothesis that soil microbes can become acclimated to triclosan at all but the very highest doses.

There are several ways in which a better understanding of the effects of triclosan on the phenotypic structure could have been examined. If the soil washing method had not been effective then other techniques for studying aspects of community structure would have been used. Studies using Community Level Physiological Profile (CLPP) techniques such as Biolog plate technology or multiple substrate induced respiration (MSIR) can also reveal changes in community structure. With both of these methods, a snapshot of the soil community can be observed. However, if more detail is needed such as species level information, more advanced molecular techniques should be employed. PLFA analysis provides a robust measure of total biomass and a broad overview of the microbial community composition grouped into general

categories such as anaerobic Gram negative bacteria. DNA based techniques such as quantitative polymerase chain reaction (qPCR) analysis (which makes copies of specific genes and quantifies the number of those target genes present in the sample) on the other hand, can quantify specific organisms and identify genes encoding specific functions. This type of analysis is best used to quantify specific bacteria and functional genes responsible for the biodegradation of organic pollutants as opposed to the microbial community structure. Other less specific DNA techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis can be used to provide a DNA based profile of the microbial community and allow identification of the predominant organisms generally to the family or genus level although they cannot quantify specific organisms or microbial functions. DGGE profiles are used to visually display differences or shifts in microbial community composition over time or in response to treatment. In the future, the work already done here could be enhanced by using some of these molecular techniques to identify specific triclosan degraders in the soil or to identify particular bacteria that are susceptible to triclosan and the impact that their absence will have on the soil overall health and function of the soil (e.g. on the nutrient cycling).

## 8.2 Conclusions

In summary, this thesis has identified and attempted to fill important knowledge gaps in the fate and effects of adding triclosan to land through the application of sewage sludge. The thesis has enhanced overall understanding of the fate and effects of “down the drain” organic compounds in general. Chapter 2 identified the formation of

the metabolite methyl-triclosan in soils, suggesting rapid primary degradation but also the rapid formation of non-extractable residues. Chapter 4 also reported the formation of methyl-triclosan in soils supporting the findings from Chapter 2 and showing that this process occurs naturally in the field environment. Chapter 4 also identified some of the key drivers for triclosan fate, including climate (soil temperature and moisture content) and soil physical and chemical properties, such as organic matter and clay content and pH. These factors also appeared to play a major role in determining the effects of triclosan in soil. For example, the inhibition of respiration and biomass was lower in heavy soils with a fine texture (Chapter 5). This was probably due to the high micropore fraction in there soils which offer more protection to organic compounds from microbes in comparison to the coarser soils. Another key finding from Chapter 5 was that respiration and biomass in soils treated with triclosan recovered to levels which were greater than or equal to those measured in control soils. In addition to the resilience observed, re-dosing the soils with triclosan resulted in a different response. In samples treated with a low triclosan dose, triclosan appeared to be utilised as a substrate, and overall toxicity was lower in all treatments suggesting there was some acclimation to triclosan.

In order to determine how the microbial community structure had been changed as a result of the acclimation, phenotypic profiling was undertaken (Chapter 7). This study showed that the community did indeed respond to triclosan dosing but it also showed that the response was a complex one with factors such as soil type, time and general laboratory treatment effects all potentially influencing changes in microbial community structure.

An important contribution of this thesis was in the design, testing and helped in the determination of the key findings. First a new analytical method was developed (Chapter 3), which was used to detect and quantify triclosan and methyl-triclosan in soil. This method is the first to simultaneously extract, detect and quantify low levels of both compounds from a soil matrix. The second method developed was the solvent washing procedure to remove triclosan from soil prior to PLFA analysis (Chapter 6). This simple method removed high triclosan concentrations from the soil allowing for accurate PLFA analysis to be carried out without modifying the microbial community structure.

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